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Arachidonic acid metabolism by the pregnant rat uterus.

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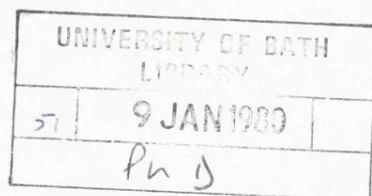
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ARACHIDONIC ACID METABOLISM

BY THE

PREGNANT RAT UTERUS

Submitted by

I. Downing, B.Sc. (Hons.)

for the degree of Doctor of Philosophy

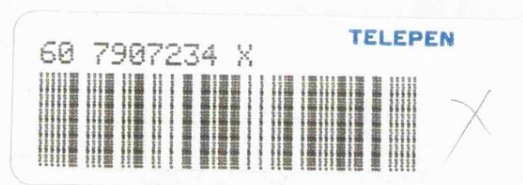
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TO MY WIFE AND PARENTS.

Summary

Prostaglandin biosynthesis by decidual and myometrial microsomes from pregnant rat uteri was investigated by incubation with $[1-^{14}\text{C}]$ -arachidonic acid. Arachidonic acid metabolites synthesized by these preparations were separated and identified using TLC procedures. The optimal incubation conditions for maximum biosynthesis were determined.

The influence of hydroquinone, GSH, L-cysteine and EDTA, used as cofactors in microsomal incubations, on overall prostaglandin synthesis was examined. The effect of SH group containing cofactors on the ratio of prostaglandins synthesized was also investigated.

The effect on prostaglandin biosynthesis by decidual and myometrial microsomes of replacing hydroquinone with other possible 'natural' cofactors was inspected. Of particular interest were biogenic amines such as adrenaline and noradrenaline, which have been postulated as possible endogenous cofactors.

The influence of the day of pregnancy on prostaglandin synthesis by decidual and myometrial microsomes was examined with respect to the quantity and type of prostaglandins synthesized. It was found between days 13-22 of pregnancy that prostaglandin biosynthesis increased as pregnancy progressed. Maximum prostaglandin synthesis was observed on day 22 of pregnancy - expected day of delivery. Synthesis declined sharply one day post-partum.

The incubation of decidual and myometrial microsomal suspensions with a selection of non-steroidal anti-inflammatory drugs showed that uterine prostaglandin synthetase was inhibited by these drugs and did not differ radically from the inhibition of other prostaglandin

synthetases. However, when inhibited by tranylcypromine or metopirone there was no preferential inhibition of one particular prostaglandin as seen by other workers. The most potent drug tested was flurbiprofen.

An inhibitor of arachidonic acid metabolism by decidual and myometrial microsomes was found in the microsomal supernatant of decidual, myometrial and placental microsomes. The inhibitor was found to be heat labile, inhibited in a dose dependent manner, and was not due to metabolism of prostaglandins, with the site of action at the cyclo-oxygenase.

ACKNOWLEDGEMENTS

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CHAPTER ONE

INTRODUCTION

1.1 History

Goldblatt (1933, 1935) and Von Euler (1935, 1937) investigating independently human seminal plasma and extracts of sheep seminal vesicular glands discovered a novel substance having both vasodepressor and smooth muscle stimulating activity. As it was found in extracts of both prostate and vesicular glands Von Euler (1935) named the principle prostaglandin. Initial characterisation established it to have the properties of a fatty acid, being easily extracted with lipid solvents from an acidic aqueous solution, also freely soluble in aqueous alkali. Further characterisation was hindered by the lack of appropriate techniques, but a little later it was shown that prostaglandin was nitrogen free, probably being unsaturated and containing hydrophilic groups. Its purification was aided by the preparation of a soluble barium salt. Von Euler (1939) concluded that prostaglandin was a lipid soluble fatty acid probably containing a double bond and a hydroxy group.

New techniques developed during the 1950's provided effective new methods for purification and separation; chromatography playing a major role. Bergström and Sjöval (1957) isolated one prostaglandin 'factor' (PGF) in crystalline form. In 1960 this was followed by the isolation and preliminary x-ray crystallography for prostaglandins F and E (Bergström and Sjövall, 1960a,b). This firmly established prostaglandins not as a single compound but a group of chemically related compounds, all derivatives of a parent substance, prostanoic acid, which is not known to occur naturally.

After some resourceful work the structure of the various prostaglandins were elicited; confirmation was obtained using x-ray crystallography (Abrahamsson, Bergström and Samuelsson, 1962) and mass spectroscopy (Bergström, Ryhage, Samuelsson and Sjövall, 1963) still

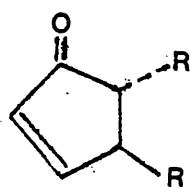
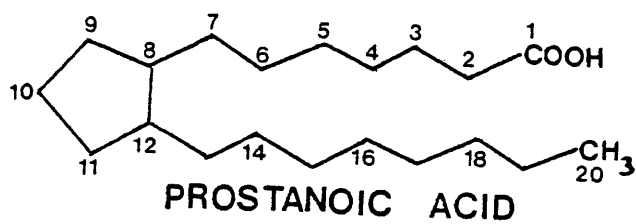
one of the most useful and powerful techniques available today. In 1964 the first biosynthesis of prostaglandins was achieved, Van Dorp, Beerthuis, Nugteren and Vonkeman (1964a) and independently Bergström, Danielsson, and Samuelsson (1964) published details of the synthesis of prostaglandin E_2 from arachidonic acid using sheep vesicular glands as a source of the enzyme. Details of the isolation and properties of intermediates in prostaglandin biosynthesis were published, independently by Nugteren and Hazelhof (1973) and Hamberg and Samuelsson (1973). The intermediates were later shown to be endoperoxides.

A few years later, while investigating arachidonic acid metabolism by washed human platelets, Hamberg, Svensson and Samuelsson (1975) found a new group of compounds also formed by the metabolism of arachidonic acid via the endoperoxide intermediates. Due to the new group of non-prostanoid compounds origin and structure they were named thromboxanes. This was shortly followed by Gryglewski, Bunting, Moncada, Flower and Vane (1976) announcing the discovery of another metabolite of the cyclic endoperoxides (now known as prostacyclin) which effectively antagonised the biological activity of the thromboxanes.

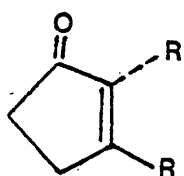
1.2 Chemistry

The prostaglandins (PGs) are unsaturated hydroxy-acids with a five membered ring in a twenty carbon atom skeleton. Their structure is based on a monocarboxylic acid given the trivial name prostanic acid, which is not known to be a naturally occurring substance. PG series are designated by letter, e.g. PGE. Each series is differentiated by the position of various functional groups or double bonds, usually occurring within the cyclopentane ring. PGG is unusual in having a hydroperoxy group at the C-15 position, all others possessing

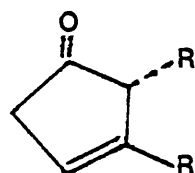
Figure 1. Numbering System of Prostanoic acid and Chemical
Structures of Prostaglandins and Thromboxanes



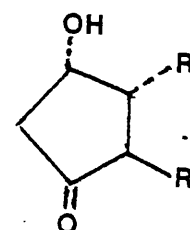
PGA



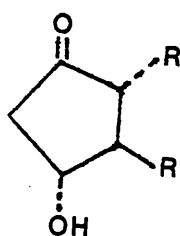
PGB



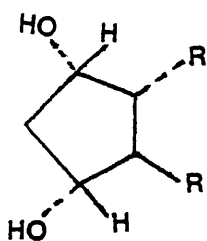
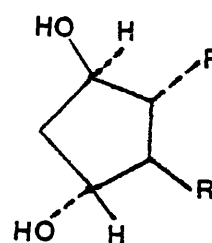
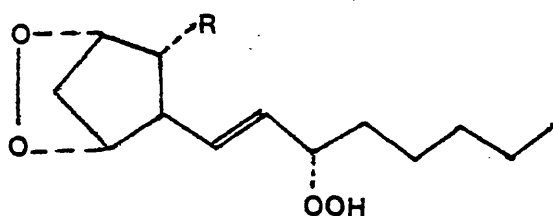
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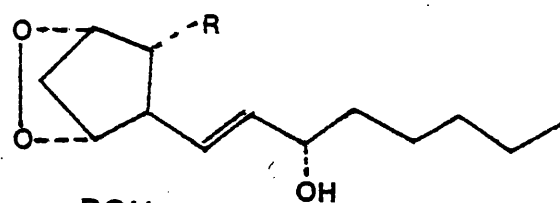
PGD



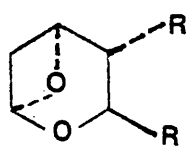
PGE

PGF α PGF β 

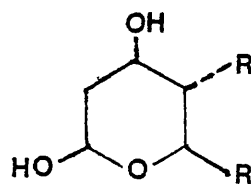
PGG



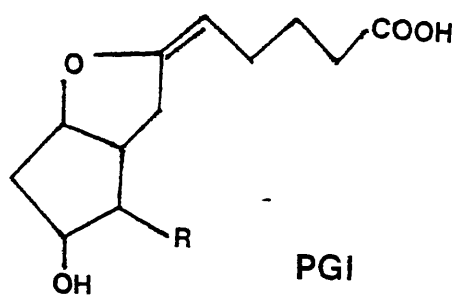
PGH



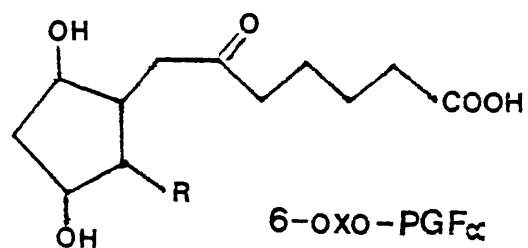
TxA



TxB



PGI

6-oxo-PGF α

a hydroxyl group in this position. Nine series have been isolated to date from PGA to PGI inclusive.

The members of each series differ by the number of double bonds they possess in the side chains. The number of double bonds in the side chains being designated by a subscript numeral, e.g. PGE_2 , which has two double bonds in its side chains. PGs of the "1" series invariably have a double bond in the trans Δ^{13-14} position, members of the "2" series have another cis double bond in the Δ^{5-6} position. The "3" series possess yet another cis double bond in the Δ^{17-18} position. In the F series of PGs an additional subscript refers to whether the C-9 hydroxyl group is above (α) or below (β) the plane of the cyclopentane ring i.e. denotes isomeric form.

The "1" and "2" series are the major PGs found in biological systems. The degree of unsaturation being determined by the fatty acid precursor. The precursor for the "1" series is all cis-8,11,14,eicosatrienoic acid (dihomo- γ -linolenic acid, DHLA) and the precursor for the "2" series is all cis-5,8,11,14,eicosatetraenoic acid (arachidonic acid, AA).

Chemically the A series of PGs are dehydration products of the E type, with the C-11 hydroxyl group being removed and a Δ^{10-11} double bond formed. The B type PG is an isomer of PGA, where the Δ^{10-11} double bond is shifted via the Δ^{11-12} position (PGC which is relatively unstable) to the Δ^{8-12} position. The transformation of PGE into PGA or PGB is easily achieved by treating PGE with acid or base. Since PGA and PGB are chromophores with characteristic U.V. absorption maxima of 217 nm and 278 nm respectively, the transformation of PGE into PGA or PGB can be used as a method of assaying PGE. However, as an assay it is relatively insensitive requiring

several μg of PGE (Bergström, Danielsson, Klenberg and Samuelsson, 1964).

PGE or PGD (which is an isomer of PGE with the C-9 keto and C-11 hydroxyl groups interchanged) is easily converted into PGF_{α} or PGF_{β} by treatment with sodium borohydride. Conversion of the endoperoxides PGG and PGH is readily achieved with a reducing agent, e.g. treatment of PGG_2 and PGH_2 with stannous chloride or triphenylphosphine yields $\text{PGF}_{2\alpha}$. The latest PG series to be discovered, PGI is a bicyclic system with a cyclopentane and furan ring. This is unstable and rapidly rearranges to 6-oxo- PGF_{α} , (Johnson, Morton, Kinner, Gorman, McGuire, Sun, Whittaker, Bunting, Salmon, Moncada and Vane, 1976).

The thromboxanes (TX) are C_{20} unsaturated hydroxy-acids but unlike prostaglandins are not based on the prostanoic acid skeleton, although they are derived from the same fatty acid precursors. The thromboxanes have an oxane ring in place of the cyclopentane ring of the PGs, but with the hydroxyl group at C-15. There are two series, TXA and TXB, the same subscript conventions applying as with PG nomenclature. TXA has a bicyclic oxane-oxetone ring system. This is unstable and when attacked by a nucleophile (e.g. water) yields the stable and inactive hemiacetal derivative TXB.

1.3 Biosynthesis of Prostaglandins

Arachidonic acid (AA) cannot be synthesized de novo by animal tissues, and linoleic acid is required as a precursor for its synthesis, this is provided by the animals diet. When the AA is synthesised and stored it is only that AA stored in membrane phospholipid pools that is available for PG synthesis. Flower and Blackwell (1976)

incorporated $[1-^{14}\text{C}]$ -AA into guinea-pig spleen and identified phospholipids as the only source of substrate for PG synthetase. Lands and Samuelsson (1968) using a sheep seminal vesical preparation found that a phospholipid substrate was only effective if the fatty acid was cleaved off, freeing the AA. The biosynthesis of PGs from AA was first demonstrated using homogenates of sheep vesicular glands as a source of the PG synthetase (Van Dorp, Beerthuis, Vorkeman, 1964a; Bergström, Danielsson and Samuelsson, 1964). The first microsomal preparation of PG synthetase was by Van Dorp, Beerthuis, Nugteren and Vorkeman (1964b), again using sheep vesicular glands as the source of PG synthetase. Figures 2 and 3 show the biosynthesis of the 2 series of PGs and TXs from AA. This is the most studied pathway as AA is far more abundant than dihomo- γ -linoleic acid.

PG synthetase is an enzyme complex. The first part, the cyclo-oxygenase component converts AA into the cyclic endoperoxides PGG_2 and PGH_2 . These can then be converted to the PG or TX required by the isomerase component (Nugteren, Beerthuis and Van Dorp, 1966; Hamberg and Samuelsson, 1967). This conversion can be divided into three distinct steps, (1) initial double bond oxygenation (cyclo-oxygenase) (Hamberg, Svensson, Wakabayashi and Samuelsson, 1974); (2) formation of the 15-hydroperoxy group of PGG (peroxidase) (O'Brien and Rahimtula, 1976) and (3) conversion of endoperoxides into PGs or TX (isomerase) (Nugteren and Hazelhof, 1973).

The initial step in the conversion of AA is the stereospecific removal of the L-hydrogen at C-13, followed by isomerisation of the 11-cis double bond to 12-trans with the simultaneous attachment of one molecule of oxygen at C-11. There then follows the attachment of further molecular oxygen at C-15, with the concerted reaction

Figure 2. Metabolism of Arachidonic Acid to PGG₂ and the alternate conversion to HETE by Cyclo-oxygenase and Lipoxygenase enzymes respectively.

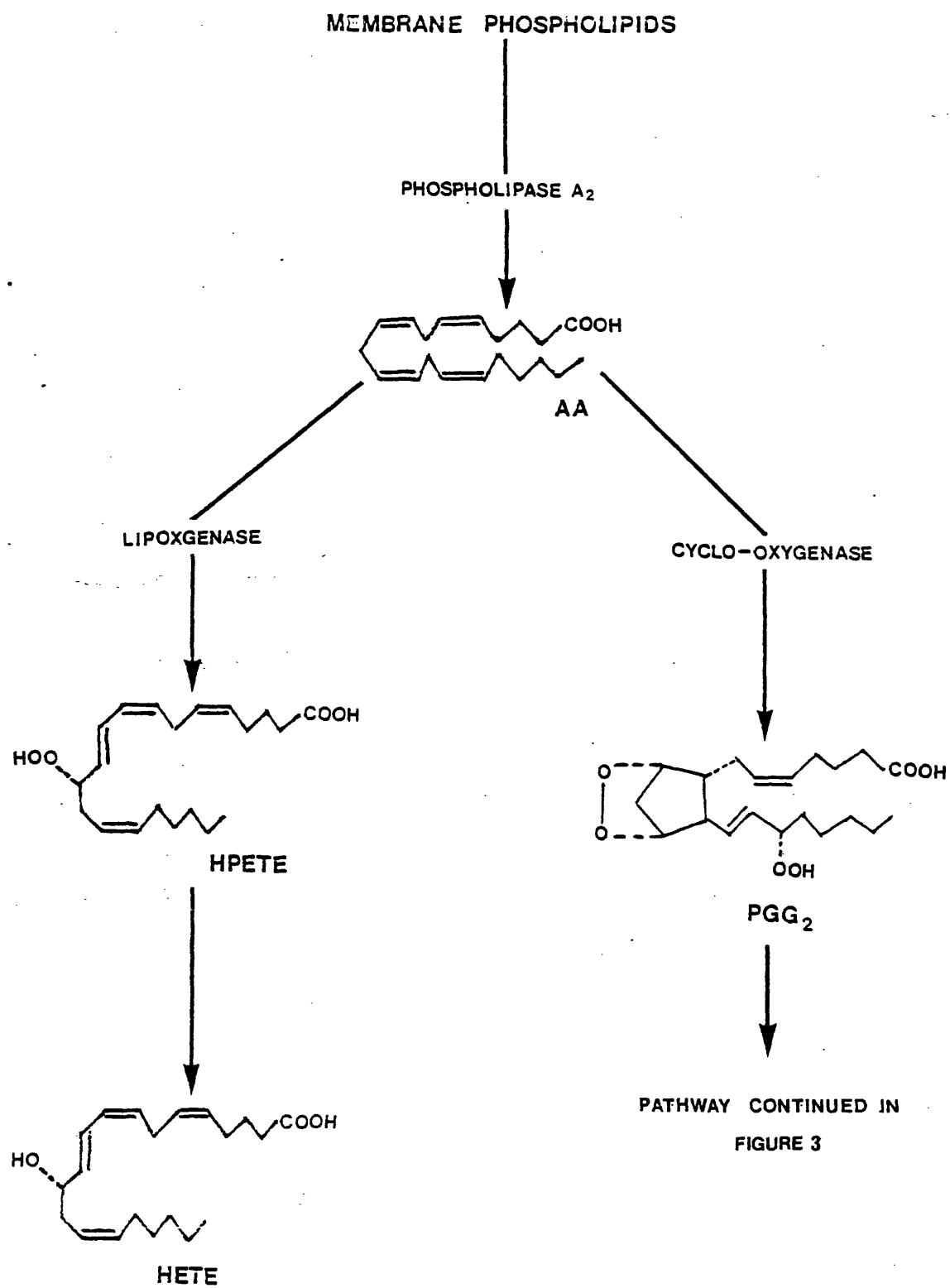
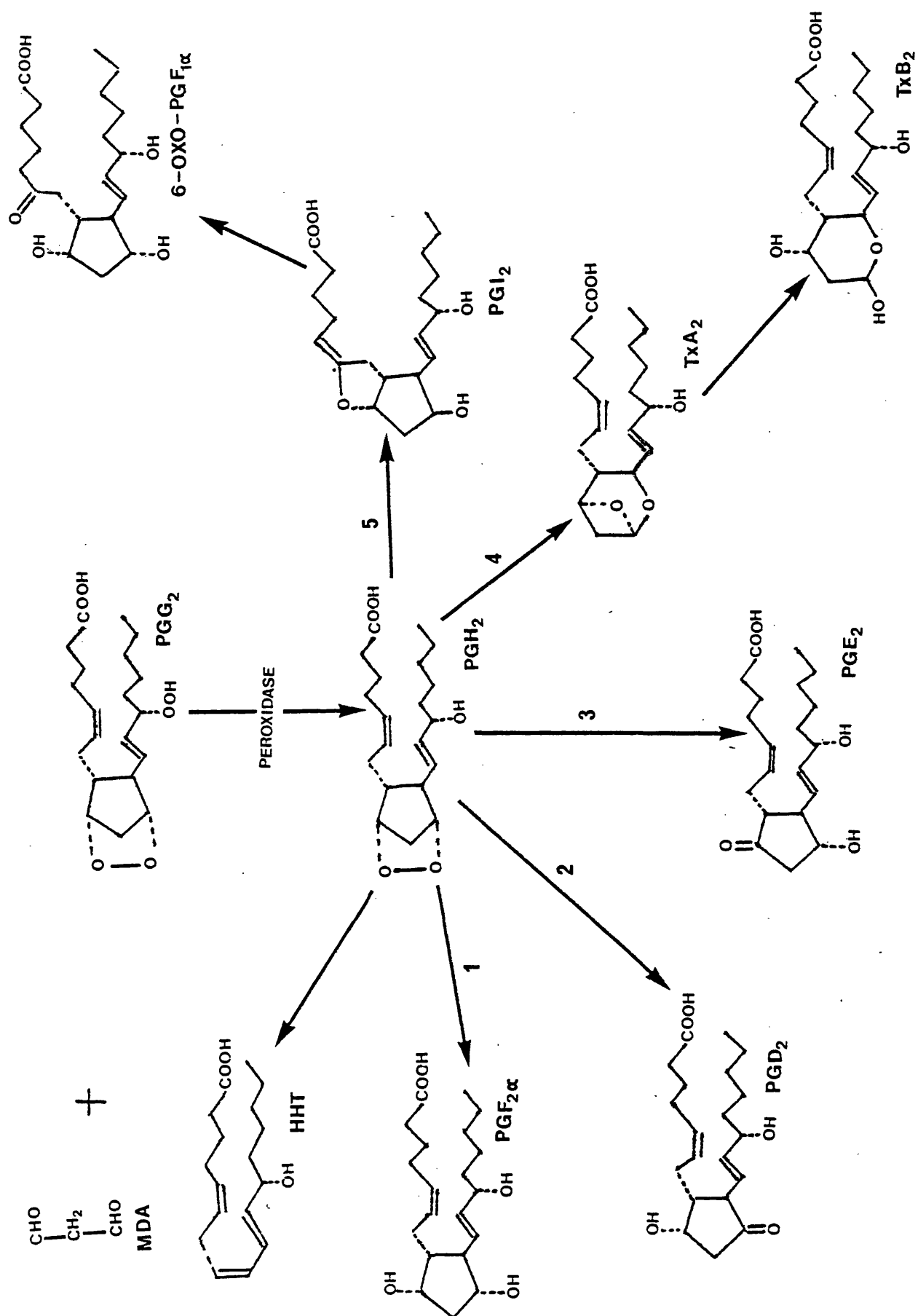


Figure 3: Metabolism of PGG_2 to PG-like products, and HTT

(formed non-enzymically).

Enzymes involved in cyclic endoperoxide metabolism

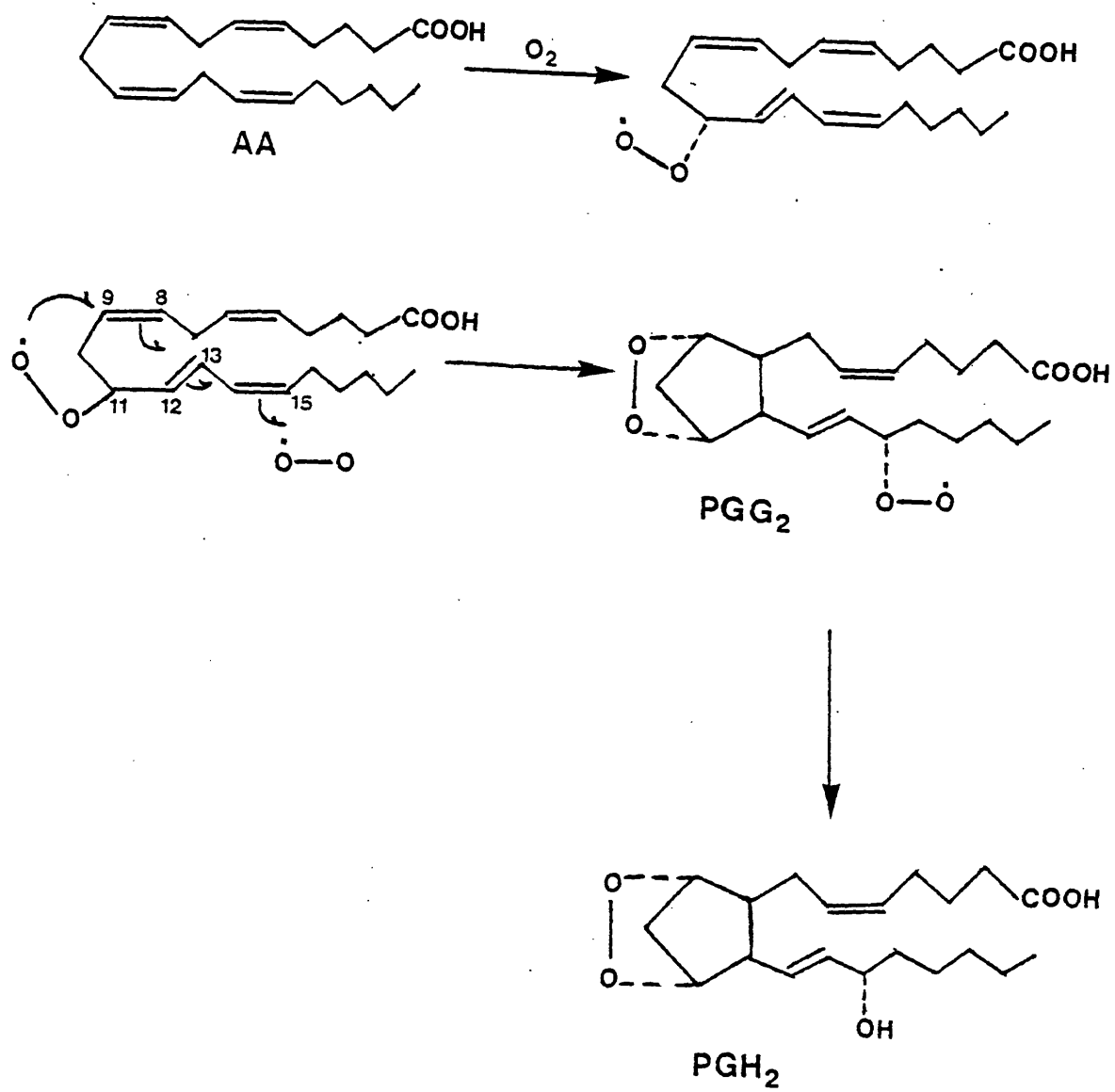
- 1) Endoperoxide reductase
- 2) PGH-PGD isomerase
- 3) Endoperoxide isomerase
- 4) Thromboxane synthetase
- 5) Prostacyclin synthetase



in which there is formation of the C-8 C-12 bond (ring closure) and attack at C-9 by the oxygen radical to form the cyclic endoperoxide PGG_2 . The peroxide at C-15 can then be degraded to a hydroxyl, yielding PGH_2 (see Fig. 4). The cyclo-oxygenase provides a single common precursor for PGE_2 and $\text{PGF}_{2\alpha}$ (Wlodawer and Samuelsson, 1973). Present knowledge indicates that the cyclic endoperoxides are the common precursors of all PGs and TXs known to date.

The transformation of AA to PGs has been postulated to involve a free radical mechanism (Samuelsson, 1967). Panganamala, Sharma, Sprecher, Geer and Cornwall (1974) carried the idea further and proposed that the enzymic breakdown of hydrogen peroxide generates an .OH radical and this is required for the initial oxygenation. In support of this Panganamala et al (1974) found that catalase (which converts hydrogen peroxide to water and molecular oxygen) inhibited PG synthetase. Thus the fact that PG biosynthesis is stimulated by hydroquinone is possibly explained by the ability of hydroquinone to generate hydrogen peroxide by a quinone-hydroquinone cycle (Deamer, Heikkila, Panganamala, Cohen and Cornwall, 1971). Also the stimulation of PG biosynthesis by Fe^{2+} enzyme complexes e.g., haemin, haemoglobin (Yoshimoto, Ito and Tomita, 1970) may be explained by the ability of Fe^{2+} complexes to catalyse the decomposition of hydrogen peroxide and generate .OH radicals. There has been an alternative mechanism proposed (Rahimtula and O'Brien, 1976) involving a peroxidase mechanism in which a singlet oxygen metal complex reacts with AA to form an unconjugated hydroperoxide. The hydroperoxide further reacts with the peroxidase part of the PG synthetase to form a peroxy radical which could then form the cyclic endoperoxide by a two step cyclization reaction.

Figure 4: Mechanism for the metabolism of AA to PGH_2



The pivotal substances in the synthetic pathway are the cyclic endoperoxides, PGG_2 , and PGH_2 ($t_{1/2}$ approximately 5 min, Needleman, Moncada, Bunting, Vane, Hamberg and Samuelsson, 1976) which may be modified into a diversity of products by the isomerase component of the PG synthetase complex. They may also spontaneously degrade to give PGE_2 , PGD_2 and $\text{PGF}_{2\alpha}$ (Sun, 1977). Enzymically, via the isomerase component the cyclic endoperoxides may yield PGE_2 or PGD_2 (by removal of the C-9 hydrogen atom or the C-11 hydrogen atom from the endoperoxide bridge respectively); reductive cleavage will yield $\text{PGF}_{2\alpha}$. A C_{17} hydroxy fatty acid (12-hydroxy-5,8,10-heptadecatrienoic acid, HHT) and malondialdehyde (MDA) can also be formed from the PGH_2 by cleavage of a carbon-carbon bond. However, these products are of minor importance as they are thought to be formed by non-enzymic thermal degradation (Pace-Asciak, 1977). Until recently the significant active end products of the cyclic endoperoxides formed by the isomerase component were thought to be PGE_2 , PGD_2 and $\text{PGF}_{2\alpha}$. But now TX synthetase, the predominant metabolic route in human platelets for AA has been identified (Hamberg, Svensson and Samuelsson, 1975; Needleman, Moncada, Bunting, Vane, Hamberg, and Samuelsson, 1976). This enzyme converts the cyclic endoperoxides to TXA_2 , a potent aggregating agent $t_{1/2}$ approximately 30 sec, (Needleman, et al, 1976) that rapidly degrades to TXB_2 , an inactive substance.

More recently another synthetase has been found in arterial walls, and stomach fundus, known as prostacyclin synthetase which converts the cyclic endoperoxides to PGI_2 a highly active anti-aggregatory agent which spontaneously decomposes ($t_{1/2}$ approximately 3 minutes, Bunting, Moncada and Vane, 1978) to an inactive metabolite, 6-oxo- $\text{PGF}_{1\alpha}$ (Johnson, Morton, Kinner, Gorman, McGuire, Sun, Whittaker,

Bunting, Salmon, Moncada and Vane, 1976).

AA can be metabolised by alternative pathways from PG synthetase, i.e. by lipoxygenase. This enzyme converts AA to 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE). Such conversion is usually small. As yet the biochemical properties of lipoxygenase have not yet been fully elucidated and the importance of these products is difficult to assess.

Purification of PG synthetase: The microsomal cell fraction was chosen as a starting point for the purification of PG synthetase. The microsomal fraction has the advantages that most of the endogenous substrate and the PG metabolising enzymes remain in the microsomal supernatant. Therefore, exogenous substrate (e.g. $[1-^{14}\text{C}]$ -AA) is not diluted and the products are not metabolised. After solubilisation of the PG synthetase with a non-ionic detergent (e.g. Tween 20, Samuelsson, 1970) an attempt was made to isolate the individual members of the PG synthetase complex obtained from bovine seminal vesicles (Miyamoto, Yamamoto and Hayaishi, 1974). Using a DEAE-cellulose column, two fractions were obtained fraction I, which (in the presence of tryptophan and haemoglobin) carried out the cyclo-oxygenase and peroxide functions of PG synthetase. Fraction II carried out the isomerase function on the product of fraction I, utilizing reduced glutathione (GSH) as a co-factor. Fraction I was found to have a molecular weight of 300-350,000 by gel filtration (Miyamoto, Ogino, Yamamoto and Hayaishi, 1976).

Roth, Stamford and Majerus (1975) used $[\text{acetyl } -^3\text{H}]$ - aspirin to label the cyclo-oxygenase and estimated the molecular weight by gel-electrophoresis. Using sheep and bovine seminal vesicles micro-

somes and human platelet microsomes as a source of the cyclo-oxygenase the molecular weight was shown to be 85,000. A later study on sheep vesicular gland microsomes by Van Der Oudraa, Buyterhek, Nugteren and Van Dorp (1972) found that the molecular weight of the cyclo-oxygenase was between 72, and 85,000, but that in Tween 20 it behaved as a dimer. This may account for the high molecular weight found by Miyamoto et al (1974).

Cofactor Requirements: Samuelsson (1967) first demonstrated that the Production of PGs by sheep seminal vesicle microsomes were markedly stimulated by a heat stable cofactor(s) in the microsomal supernatants. This cofactor(s) could be replaced by exogenous cofactors in vitro because a specific stimulation was also achieved with GSH and anti-oxidants such as hydroquinone and propylgallate (Nugteren, Beerthuis and Van Dorp, 1966). As microsomes are used to generate PGs and the cofactors are contained in the supernatant it is usual practice to add cofactors exogenously. Examples are GSH and hydroquinone or adrenaline which supply the reducing equivalents required by PG or TX synthetase. GSH may be one of the natural cofactors for some synthetase systems as several other thiol compounds failed to stimulate PG biosynthesis (Van Dorp, 1967). However, it must also be taken into account that some systems appear to function without GSH. Cottee, Flower, Moncada, Salmon and Vane, (1977) found that ram seminal vesicle microsomes incubated in the presence of GSH produced a decrease in 6-oxo-PGF_{1α} formation and an increase in PGE₂ formation. In the absence of GSH only 6-oxo-PGF_{1α} was formed. GSH may be required by the PGE isomerase component of the enzyme, or alternatively may act non-enzymically directly on the cyclic endoperoxides. Lee and Lands (1972) showed that a copper-dithiol complex would facilitate the formation of PGF over PGE, perhaps by supplying the cyclic endoperoxide with a source of electrons.

It has also been shown that haem compounds (e.g. haemoglobin, myoglobin and haemin) stimulate PG biosynthesis. Yoshimoto, Ito and Tomita (1970) concluded that haem compounds and hydroquinone were involved in the attachment of molecular oxygen to the fatty acid substrate whereas GSH was utilized for the reduction of the cyclic endoperoxide. However, Sih, Takeguchi and Foss (1970) proposed that GSH supplies reducing equivalents for the reduction of the oxidized hydroquinone, perhaps via an enzymic mechanism.

Another cofactor to be considered is L-tryptophan, proposed to stimulate formation of the cyclic endoperoxides in the presence of haem (Miyamoto, Yamamoto and Hayaishi, 1974), the cyclo-oxygenase requiring both cofactors for synthesis. The system used also required GSH for conversion of the cyclo-oxygenase into PGE or PGF.

Control of PG Biosynthesis: It has been found that there is little free fatty acid substrate available for PG synthetase to utilize. Furthermore, PGs are not stored in cells as the dog spleen contains less than 1µg of PGs but can release 10µg in the first minute after stimulation (Piper and Vane, 1971). Samuelsson (1972) postulated that control of PG biosynthesis could be achieved by controlling the access of the substrate to the PG synthetase. This would enable humoral or neuronal stimuli to act as a trigger for PG biosynthesis. Flower and Blackwell (1976) confirmed that the phospholipids are the source of AA for PG synthetase. Therefore, the release of AA by phospholipase A₂ is the rate limiting step in PG production (Bosisio, Galli, Galli, Nicosia, Spagnuolo, and Tosi, 1976).

There are two possible mechanisms by which phospholipase A₂ may be activated. The first is by control of free calcium ions since plasma membrane and microsomal phospholipase activity is totally

dependent on the presence of free calcium ions (Newkirk and Waite, 1973). It is phospholipase A_2 from these sources that is implicated in cleaning the substrate required by the PG synthetase (Vogt, 1978). Several substances which increase PG production are known to enhance calcium ion penetration of cell (e.g. catecholamines). The second mechanism of control of phospholipase A_2 activity may depend on access of the enzyme to the phospholipid, which is affected by the structural arrangement of the phospholipid in the bilayer membrane (Verger, Rietsch, Van Dam-Mieras and De Haass, 1976). It has been claimed that the incorporation of foreign molecules into a membrane (e.g. local anaesthetics in low concentrations) will enhance membrane phospholipid cleavage, and increase PG synthesis (Vogt, 1978). It is possible that a stimulant (e.g. hormone) interacting with a receptor may change the permeability of the membrane to phospholipase A_2 .

1.4 Metabolism of Prostaglandins

With the introduction of tritiated PGs it was possible to follow the metabolic fate of PGs in vivo. The most important step for biological inactivation is the oxidation of the hydroxyl group at C-15. The enzyme responsible for this is PG 15-hydroxy dehydrogenase (PGDH) and gives rise to the 15-keto PG. This metabolite can be further metabolised by reduction of the Δ^{13} double bond by PG Δ^{13} reductase. This is then followed by both a β and ω oxidation. The enzymes PGDH and PG Δ^{13} reductase are both found in the soluble cytoplasmic fraction of tissue homogenates and both require NADH as a cofactor. It has been shown that the substrate for PG Δ^{13} reductase must previously have had the C-15 hydroxyl group oxidised to activate the double bond (Samuelsson, Granström, Green and Hamberg, 1971).

There is a difference in the end product of PG metabolism between species (normally occurring after the PGDH and PG Δ^{13} reductase step). But, the prime sites for the metabolism of circulating PGs in the bloodstream for all species are the lung and liver. It has been shown that a single passage through the lungs of a cat, dog, or rabbit caused a 90-95% loss of the biological potency of PGs and a single circulation through the liver caused a 70-93% loss (Ferreira and Vane, 1967).

Reproductive tissue has a well documented ability to metabolise PGs, employing predominantly PGDH and PG Δ^{13} reductase to deactivate PGs (Keirse and Turnbull, 1975). Virtually all uterine tissues appear to have some metabolic capacity, which is particularly high in foetal membranes and placenta, followed by the myometrium and decidua, while only low activity has been detected in the umbilical cord and none has been demonstrated in either amniotic fluid or umbilical venous blood (Keirse, 1978). It has also been demonstrated that in rabbits during pregnancy there is a marked increase in PGDH activity in the rabbit lung (Egerton-Vernon and Bedwani, 1975). Blackwell, Flower and Vane (1975) showed that PGDH was a short-lived enzyme, by easily diminishing the ability of the kidney and lung to metabolise PGs with one dose of a protein synthetase inhibitor. They also postulated that PGDH synthesis may be controlled by steroid or hormone factors circulating in the blood stream. Bedwani and Marley (1975) had already shown that in rabbits progesterone treatment enhanced PGDH activity. Blackwell and Flower (1976) went on to show that hydrocortisone hemisuccinate and dexamethasone could reduce PGDH activity in adrenalectomized rats, and further postulated that the decrease in PGDH levels seen at parturition could be due to the oestrogen surge which occurs approximately that time.

Apart from PGs being deactivated by metabolism there are enzymes which will convert one active PG into another. A series of conversions occur in serum, PGE to PGA by a dehydrogenase followed by conversion of PGA to PGC by PGA isomerase, and finally conversion of PGC to PGB via PGC isomerase (Polet and Levine, 1975). The conversion of PGE to PGF has been demonstrated in the cytoplasmic fraction of guinea-pig liver (Hamberg and Israelsson, 1970) and sheeps blood (Hensby, 1974a). Transformation of PGD_2 to $\text{PGF}_{2\alpha}$ by a PG-11-keto-reductase has also been reported (Hensby, 1974b). There is a report of conversion of $\text{PGF}_{2\alpha}$ to PGE_2 in rat kidney by PG-9-hydroxydehydrogenase (Pace-Asciak, 1975). Finally there is the possibility of non-enzymic conversion, normally to inactive metabolites i.e. degradation of TXA_2 to TXB_2 , or degradation of PGI_2 to 6-oxo- $\text{PGF}_{1\alpha}$ (although in vivo it is suggested these substances are substrates for PGDH, McGuire and Sun, 1978). The ability of PGs to undergo non-enzymic conversion must be considered especially during extraction procedures. The major example being the controversy over the possibility that PGA detected in renal medulla homogenates may have been a degradation product of PGE created by the isolation and extraction procedure (Frolich, Sweetman, Carr, Hallifield and Oates, 1975).

1.5 Measurement of Prostaglandins

There are two approaches to the measurement of PGs in biological samples. The first is to extract the endogenous PGs present and assay them by one of the accepted techniques. However, the quantities extracted are normally low. Flower and McClure (1975) state that generally quantities are between 0.1-1 $\mu\text{g/g}$. At these concentrations spect roscopic and optical techniques are not sufficiently sensitive, and cofactors, may also interfere with measurement

depending on absorption maxima at particular wavelengths.

A much used technique of estimation is bioassay, using a superfused cascade with suitable tissues (Gilmore, Vane and Wyllie, 1968). This method provides adequate sensitivity and with the use of antagonists can be rendered selective. However, this system cannot be used to detect metabolites of active PGs as these have little activity. Bioassay is useful for detecting labile substances like TXA_2 and PGI_2 , which are easily assayed directly after release from a tissue.

Radioimmunoassay is probably the most sensitive technique, with a threshold of 10-15 pg (Bauminger, Zor and Lindner, 1973). This method is most suited to A,B or F type PGs as the chemical manipulation required to attach PGE to its carrier protein can result in conversion to PGA. The PGA subsequently, in vivo can be isomerised to PGB, producing a mixed antibody preparation. Another disadvantage is that radioimmunoassay measures the inhibition of the binding of a labelled antigen to antibody, and this inhibition can be caused by other factors than PGs, i.e. pH, ionic strength, protein concentration (Granström, 1978). Gas chromatography/mass spectroscopy techniques can deal with ng quantities but this requires careful derivatisation, expensive equipment and experience to operate efficiently.

The alternative to measuring endogenous levels of PGs is to measure the PG synthesizing capacity of a tissue. The isolated tissue to be studied is firstly homogenised. If only a crude homogenate is prepared, there is the advantage that the endogenous cofactor(s) is present, but so is the endogenous substrate, usually an unknown quantity. If a microsomal preparation of the tissue is

made then there is the disadvantage that the endogenous cofactor(s) have to be replaced by exogenous cofactors. However, this system has the advantage that the endogenous substrate and PG metabolising enzymes are left in the microsomal supernatant. Therefore, a radio-labelled substrate can be introduced to produce radiolabelled PGs which can be simply assayed by thin layer chromatographic techniques. Estimates of the PG synthetic capacity of a tissue may be more accurate if isolated tissue is used as it has been shown that damaged cells release PGs and can artificially elevate tissue levels (Piper and Vane, 1971; Williams, Sneddon and Harney, 1974).

1.6 The Inhibition of Prostaglandin Biosynthesis

Vane (1971) was the first to suggest that the anti-inflammatory, antipyretic, and analgesic properties of drugs such as aspirin and indomethacin (non-steroidal anti-inflammatory drugs, NSAID) was related to their ability to inhibit PG biosynthesis. That paper and those published with it (Smith and Willis, 1971; Ferreira, Moncada and Vane, 1971) paved the way for the explosion in PG research that followed.

NSAIDs appear to act on the cyclo-oxygenase component of prostaglandin synthetase. Vane (1971) showed that aspirin and indomethacin inhibited PG synthesis by guinea-pig lung homogenates in the presence of exogenous AA. Therefore, it is apparent that the site of action of NSAIDs is at a stage after the release of the free substrate. It has also been shown that indomethacin will inhibit the formation of the cyclic endoperoxide precursors of PGs by bovine vesicular gland microsomes (Miyamoto, Ogino, Yamamoto and Hayaishi, 1976). This leaves the cyclo-oxygenase as the site of action of NSAIDs. The kinetics of PG synthetase inhibition by NSAIDs is complicated by

NSAIDs being collection of drugs with similar actions rather than similar structures. While some NSAIDs are simple competitive inhibitors, other also have a time dependant irreversible inhibitor component (Cushman and Cheung, 1975). The different NSAIDs may also have different sites of action on the cyclo-oxygenase. Using $[\text{acetyl-}^3\text{H}]$ -aspirin it has been shown that aspirin will irreversibly acetylate a single protein, probably at the active site of the cyclo-oxygenase, (Roth, Stanford and Majerus, 1975). While $[2\text{-}^{14}\text{C}]$ -indomethacin has been shown not to form a covalent link with the cyclo-oxygenase, implying that the inhibition is reversible (Stanford, Roth, Shen and Majerus, 1977). This could indicate that there is a secondary effect which irreversibly inactivates the synthetase, as it has been shown in some preparations that indomethacin inhibition of PG synthetase is time-dependant and irreversible (Rome and Lands, 1965; Smith and Lands, 1971).

It has been noted that PG synthetase preparations from some sources are more susceptible to inhibition by NSAIDs than from other sources: their biochemical profiles also differ (Flower and Vane, 1974). This could be due to PG synthetase existing as isoenzymes both in different tissues from the same animal and between species. For instance Flower and Vane (1972) found paracetamol was much more active against rabbit brain synthetase than dog spleen synthetase. Also PG synthetase prepared from different rabbit tissues exhibited differing sensitivities to the inhibitory effects of indomethacin (Bhattacharjee and Eakins, 1973). This lends itself to the idea that selective synthetase inhibitors may be developed to act in specific tissues. Because of their opposing effects on blood vessels and platelet activity selective inhibitors of TXA_2 and PGI_2 synthetase are being developed. One such drug is imidazole which will inhibit the conversion of cyclic

endoperoxides to TXA_2 by platelets, while only affecting the cyclo-oxygenase at much higher concentrations than those required for its selective effect (Moncada, Bunting, Mullane, Thorogood and Vane, 1977). A similar inhibition has been noted for prostacyclin synthetase with 15-hydroxy-peroxy arachidonic acid (Moncada, Gryglewski, Bunting and Vane, 1976). This may indicate that prostacyclin synthetase may be inhibited in vivo by lipid peroxides associated with atherosclerosis and vitamin E deficiency (Moncada, Gryglewski, Bunting and Vane, 1976).

It must be noted that NSAIDs will not only inhibit PG biosynthesis but will also affect many other biological systems, indomethacin, for example will inhibit several proteolytic enzymes, histamine decarboxylase, Mucopolysaccharide biosynthesis, uncouple oxidative phosphorylation, and stabilize serum proteins, erythrocytes, and lysosomal membranes. This list is not exhaustive, for a fuller exposition see Shen (1972), and Famaey, Brooks and Dick (1975). In general for effects on these systems a higher concentration of drug is required than that for inhibition of PG biosynthesis (Ferreira and Vane, 1974). While it can be demonstrated that sufficient concentrations of the drug can be found in vivo for the realistic inhibition of PG biosynthesis (Vane, 1974; Dembinska, Grodzinska and Piotrowicz, 1974).

There are other classes of compounds apart from NSAIDs that will inhibit PG biosynthesis. Some primary examples are given below. Anti-inflammatory corticosteroids have been postulated to work by stabilizing the cell membrane and preventing access of the phospholipase A_2 to the phospholipid (Hong and Levine, 1976). It now appears that both RNA and protein synthesis is required for inhibition of PG biosynthesis (Danon and Assouline, 1978). So it may be that the corticosteroids generate a peptide which in turn stabilize the

cellular membrane. Mepacrine, an anti-inflammatory and antimalarial agent is frequently used for inhibition of phospholipase A₂ activity thereby preventing release of substrate (Vargaftig and Hai, 1972).

Some psychotropic drugs (monoamine oxidase inhibitors and tri-cyclic anti-depressants) have an inhibitory effect on prostaglandin biosynthesis. But this does not appear to be essential for their psychotropic effect (Lee, 1974; Krupp and Wesp, 1974; Kunze, Bohn, and Bahrke, 1975; Bekemeir, Giessler and Vogel, 1977). The mechanism of inhibition by psychotropic drugs is unknown.

Polyunsaturated fatty acids are another group of inhibitors. Ziboh and co-workers (Ziboh, 1973; Ziboh, McElligatt and Hsia, 1973) found that fatty acids such as ¹linolenic and eicosatrienoic acid markedly inhibited PG synthesis by microsomal preparations of human epidermis. Lands and co-workers (Lands, Le Tellier, Rome and Vanderhoek, 1972; Vanderhoek and Lands, 1973) found acetylenic fatty acids potent inhibitors and postulated that they destroyed the catalytic site of the cyclo-oxygenase and that the non-substrate fatty acids may be released with the substrate (Lands, Le Tellier, Rome and Vanderhoek, 1973). In the same paper they propose that the actual fatty acid substrates may cause self-catalysed destruction of the active site in the cyclo-oxygenase.

Some heavy metal ions have inhibitory activity. Zn^{2+} , Cd^{2+} , Cu^{2+} have been shown to be inhibitory in concentrations of $5 \cdot 10^{-5} \text{M}$, but this can be partially reversed by GSH (Nugteren, Berthuis and Van Dorp, 1966).

1.7 Prostaglandins and Uterine Function

(i) The non-pregnant uterus: PGs have been identified in the uterine tissue of many species, e.g. sheep (Wilson, Cenedella, Butcher and Inskeep, 1971), guinea-pigs (Poyser, 1972), hamsters (Saksena and Harper, 1972), rats (Saksena and Harper, 1972) and human (Downie, Poyser and Wunderlich, 1974). In sheep the principal uterine PG identified was $\text{PGF}_{2\alpha}$ with very small amounts of $\text{PGF}_{1\alpha}$, E_1 and E_2 (Wilson et al, 1971). In the guinea-pig uterus $\text{PGF}_{2\alpha}$ was again the major metabolite detected, with PGE_2 present in small quantities. In the rat both $\text{PGF}_{2\alpha}$ and PGE_2 were again detected (Ham, Cirillo, Zanetti and Kuehl, 1975), again the quantity of $\text{PGF}_{2\alpha}$ being higher than PGE_2 . In curettings of human endometrium Downie et al (1974) found $\text{PGF}_{2\alpha}$ and PGE_2 , with the quantities of $\text{PGF}_{2\alpha}$ being greater than that of PGE_2 at all times throughout the cycle.

In the study of some of the above uterine tissues a variation in quantity of PGs was noted with the changes in the menstrual cycle, for instance Downie et al (1974) found in the human endometrium that the levels of PGs fluctuated during the menstrual cycle, increasing in stages as the cycle progressed. A similar rise in PGs was also noticed during the last few days of the guinea-pig oestrus cycle which coincided with increasing oestrogen and decreasing progesterone levels in ovarian venous blood (Blatchley, Donovan, Horton and Poyser, 1972).

This suggested PG synthesis may be under hormonal control. Further evidence has been provided by Ham et al (1975) who showed for the rat that maximum levels of PGF were at proestrus, when oestrogen levels are maximal, and that a similar maxima could be induced in ovariectomized rats with oestradiol- 17β . It has also been shown

that in ovariectomized rats oestradiol-17 β alone is sufficient to increase PG levels over untreated animals (Sharma and Garg, 1972). These results support the idea that PG synthesis is under hormonal control, and that oestrogen plays a prominent role in this control.

The role of the PGs in the uterus appears to be to induce contractions. The principal effect of PGF_{2 α} (and PGF_{1 α}) in vitro is to weakly stimulate uterine contractions in mid-cycle strips of human myometrium, more vigorously on strips taken late in the menstrual cycle (Bygdeman and Eliasson, 1963a; Pickles and Hall, 1963). In vitro PGE₂ (also PGE₁ and PGE₃) normally cause a decrease in the frequency and amplitude of myometrial contractions (Bygdeman and Eliasson, 1963a), but if the myometrial strip is taken early or late in the cycle, then a slight stimulation can be evoked (Bygdeman and Eliasson, 1963b; Pickles and Hall, 1963). In vivo PGF_{2 α} retains its stimulatory effect on the uterus (Pickles, Hall, Clegg and Sullivan, 1966), while PGE₂ in vivo causes stimulation of the non-pregnant uterus (Karim, Hillier, Somers and Toussell, 1971).

The finding of PGs in endometrial curettings led Pickles, Hall, Best and Smith (1965) to postulate that PGs synthesized by the endometrium stimulate myometrial contractions which result in the sloughing off the endometrium. They further postulated that the ratio of PGF_{2 α} to PGE₂ may be crucial and a disparity in the ratio may be the cause of dysmenorrhoea. This postulate has recently received support from Lundstrom and Green (1978) who compared endogenous levels of PGF_{2 α} and its main metabolites in normal and dysmenorrhoeic women and found the PG levels were higher in dysmenorrhoeic women during menstruation. They also reported the

successful treatment of dysmenorrhoeic symptoms with NSAIDs. This supports the concept that increased PG synthesis accompanied by increased uterine activity is the major pathological mechanism of dysmenorrhoea.

Evidence is accumulating that $\text{PGF}_{2\alpha}$ may be the luteolytic factor for some species, e.g. sheep (Chamley, Buckmaster, Cain, Cerini, Cerini, Cumming and Goding, 1972); horse (Douglas and Ginther, 1972); cows (Lauderdale, 1972); and rodent (Labhsetwar, 1974). In the primates however, $\text{PGF}_{2\alpha}$ does not appear to be the luteolytic substance. It has been suggested that for the human there may be no luteolytic factor, as in hysterectomized women the menstrual cycle persists (Pharris and Shaw, 1974). It must be noted that decidualization occurs as a normal part of the menstrual cycle in humans, while in many species it occurs only on implantation of the ova. Thus in species where decidualization is a regular part of the cycle it is probable that menstruation occurs as a result of hormone changes initiated by luteal regression.

(ii) Prostaglandins and the pregnant uterus: In vitro it has been found that E and F type PGs promote contractions in strips of pregnant human myometrium (Embrey and Morrison, 1969). It was soon found that PGE_2 or $\text{PGF}_{2\alpha}$ given intravenously stimulated uterine contractions (Embrey, 1969). In fact PGE_1 , PGE_2 , $\text{PGF}_{1\alpha}$ and $\text{PGF}_{2\alpha}$ are all able to stimulate the myometrium during all stages of pregnancy PGE_2 being more potent than $\text{PGF}_{2\alpha}$. The quantities of both required to initiate contractions early in the pregnancy being 5-10 times higher than at term. There is strong evidence that PGs play a significant role in parturition. Several studies using PG synthetase inhibitors (e.g. aspirin and indomethacin) have shown that it is possible to inhibit contractions of pregnant rat uteri in vitro

(Vane and Williams, 1973); delay parturition and prolong labour in rats (Aiken, 1972; Chester, Dukes, Slater and Walpole, 1972); rabbits (Herfelendy, 1973); monkeys (Novy, Cook and Mavough, 1974) and humans (Lewis and Schulman, 1973). Furthermore indomethacin can delay premature labour (Zuckerman, Reiss and Rubinstein, 1974). While if the PG precursor AA is administered intra-amniotically it will initiate uterine contraction and promote abortion (Macdonald, Schultz and Duenhoelter, 1974).

The mechanism of the release of PGs at parturition is still unknown. It was thought at first, on finding the fall in progesterone and the concomitant rise in oestrogen levels that parturition was triggered by the alteration of oestrogen - progesterone ratio (Robson, 1933). However, it was shown that additional exogenous oestrogen did not promote abortion if administered to the pregnant rabbit (Schofield, 1962). Csapo put forward the idea that uterine activity was suppressed by progesterone, with the level of progesterone rising to cope with the lowering threshold for uterine stimulation (Csapo, 1956; 1975). Thus it is the progesterone withdrawal that precipitates parturition. Recently it has been shown in pregnant sheep that a temporary decline in progesterone levels is sufficient to initiate parturition (Mitchell and Flint, 1977). It is thought there is local control over parturition (Mitchell, Keirse, Anderson and Turnbull, 1976) and that the foetus may play a role in it (Parnham, Sneddon and Williams, 1975). It has been postulated that the release of a corticosteroid may trigger the drop in progesterone and rise in oestrogen levels, followed by rising prostaglandin levels. This is based on the results of Liggins, Grieves, Kendall and Knox (1972) who showed that infusion of dexamethasone in pregnant sheep caused an increase in $\text{PGF}_{2\alpha}$ levels and onset of labour.

Because of their ability to instigate uterine contractions at any time during pregnancy PGs are now extensively used for abortion during the second and third trimester of pregnancy, and induction of labour at term. Because PGs are rapidly metabolised analogues are being or have been developed, the most successful so far is 15-methyl-PGF_{2α}. These agents tend to be used only for induction of abortions, as it is not known if their prolonged action may harm the foetus, if used to induce labour (Thiery and Amy, 1977). The mechanism appears to be a direct stimulation of the myometrium rather than acting on the progesterone levels (Pharris and Shaw, 1974).

Other methods of abortion also appear to involve PGs. Abortion induced by hypertonic saline administration is found to increase the concentration of PGF_{2α} (Gustavii and Green, 1972). It is proposed that the saline introduced into the amniotic sac causes decidual cell damage, and as mentioned earlier (see section 1.5) damaged cells release PGs. The released PGs would stimulate uterine contractions and expel the uterine contents (Gustavii and Brunk, 1972). Abortion can also be induced by distension with physiological saline (Csapo, 1973). If the uterus of a post-partum rabbit is distended in situ mechanically by a balloon there is an increase in uterine contractions during application of the stimulus. The increase in uterine activity was associated with an increase in PGF equivalents released into the uterine venous blood (Csapo, 1973). The PG level as well as uterine activity decreased on removal of the stimulus. It has been shown that if a tissue is distorted then PGs are released (Piper and Vane, 1971). It has also been proposed that in spontaneous abortion PGs are responsible for the increase in uterine activity. Karim and Hillier, (1970) found PGF_{2α} and PGE₂ present in amniotic fluid during spontaneous abortion, but no PGs were present if pregnancy was terminated surgically.

Recently the myometrium has been shown to synthesize significant amounts of prostacyclin, but its physiological role is unknown. One function suggested has been to ensure a sufficient blood supply to the foetus (Williams, Dembinska-Kiec, Zmuda and Gryglewski, 1978), as PGI_2 is a potent vasodilator.

Thus it appears that uterine PGs may play two roles in pregnancy as shown by the fact indomethacin treated animals have a longer gestation period and a prolonged labour. Delay of parturition and lengthening of gestation is probably due to the drug preventing uterine PG synthesis which may prevent the luteolytic action of the PGs, and prevent withdrawal of the progesterone block (Fuchs, Smitasiri and Chantharaksri, 1976) although this probably does not apply to primates where PGs are not luteolytic and rhesus monkeys administered indomethacin from day 150 until delivery showed no significant elevation of progesterone levels (Novy, 1978). A second hypothesis is that the uterus does not become responsive to oxytocin unless sensitized by PG first (Schulman, 1977) the synthesis of which is prevented by the indomethacin. Prolongation of labour can be explained by the indomethacin preventing the large increase in PG synthesis seen at the time of delivery (Thorburn, Nicol, Bassett, Schutt and Cox, 1972) which contributes to the increased uterine activity. There are discrepancies between in vitro and in vivo studies, for instance Fuchs has shown that administration of indomethacin to pregnant rats near term did not inhibit uterine contractility in vivo (Fuchs, Smitasiri and Chantharaksri, 1976). Despite this paradox the studies utilizing PG biosynthesis and contractility in the pregnant rat uterus in vitro have provided a rational basis for the use of PG synthetase inhibitors to control uterine motility in the human in vivo. This is evidenced by the

fact that from these experiments indomethacin has been used to delay premature labour (Zuckerman, Reiss, and Rubinstein, 1974); women taking aspirin show extended pregnancy and period of labour (Lewis and Schulman, 1973).

Despite the physiological and clinical importance of uterine PG synthetase, particularly in parturition there is little information available concerning the uterine prostaglandin synthetase system or factors affecting it. Such a study is of essential importance and particularly in view of the fact that the potency of PG synthetase inhibition by a given aspirin-like drug varies widely between tissues (Dembinska-Kiec, Grodzinska and Piotronicz, 1974).

1.8 Aims of this Study

1. To determine optimal conditions for AA metabolism by microsomal preparations of decidual and myometrial tissue from pregnant rat uteri.
2. To identify the PG-like products generated by decidual and myometrial microsomes from exogenous $[1-^{14}\text{C}]$ -AA.
3. To study the effect of different cofactors on production of PGs by decidual and myometrial microsomes.
4. To investigate the influence of the stage of pregnancy on AA metabolism by uterine microsomes.
5. To investigate the effect of non-steroidal anti-inflammatory drugs on PG biosynthesis by decidual and myometrial microsomes.
6. To investigate the metabolism of PGs by the decidual and myometrial tissue fractions of day 22 pregnant rat uteri.

CHAPTER TWO

METHODOLOGY

2.1 Introduction

A variety of techniques are available for the identification and quantitation of PGs. Some of the most prominent techniques have been discussed in Chapter I (Section 1.5). The choice of assay usually decided on is a compromise between accuracy, sensitivity, specificity and cost.

As this study was an examination of the PG synthetase present in uterine tissue rather than a measurement of endogenous PG formation, a radiometric assay was to be employed. This would involve the incubation of PG synthetase preparations of uterine tissue with a radiolabelled substrate; extraction of products and unconverted substrate; separation by chromatographic techniques and quantitation of the products by liquid scintillation counting.

For this study it was found that the $[1-^{14}\text{C}]$ -AA available had a sufficient specific activity to detect the labelled products.

2.2. Materials

Reagents and solvents were purchased from BDH Chemicals Ltd., Poole, or the Sigma Chemical Company, London. Where possible they were of analar grade. Radiochemicals were obtained from the Radiochemical Centre, Amersham, these were:-

$[1-^{14}\text{C}]$ -arachidonic acid	specific activity	60mCi/mmol
$[5,6(n)-^3\text{H}]$ -prostaglandin E_1	specific activity	60Ci/mmol
$[5,6,8,11,12,14,15, (n)-^3\text{H}]$ -prostaglandin $\text{F}_{2\alpha}$	specific	
	activity	120Ci/mmol.

The $[1-^{14}\text{C}]$ -AA was made up to $1\mu\text{Ci/ml}$ in redistilled benzene and stored in dark sealed ampoules under nitrogen at 4°C . Unlabelled AA (grade 1 from porcine liver) stock solutions were also prepared

in redistilled benzene at concentrations of 10mg/ml and 0.1mg/ml. These were again stored in dark sealed ampoules, but at -20°C . When AA was required for use the benzene was evaporated under nitrogen at 37°C , and the AA redissolved in 30-50 μl of absolute alcohol. This was then diluted to the required concentration (so that the concentration of alcohol did not exceed 1%) with tris buffer (pH 8.0, 0.1M) containing sucrose (0.25M), hydroquinone (0.5mM), GSH (2mM), EDTA (1mM), L-cysteine hydrochloride (1mM). This buffer was used routinely throughout this study for the incubation of uterine microsomal preparations. The tritiated PGs were made up in an ethanol/water solution (7:3) at a concentration of 10 $\mu\text{Ci/ml}$ and stored in sealed ampoules at -20°C . When needed aliquots were diluted with the above buffer to the required concentration.

PPO and dimethyl POPOP were supplied by Koch-Light Laboratories, Colnbrook. Initially the scintillant used was 0.5% PPO, and 0.03% dimethyl POPOP in toluene but this was shortly changed to 0.3% PPO, 0.02% dimethyl POPOP in xylene/triton X114, (3:1), a cheaper, more efficient and versatile scintillant.

The prostaglandin standards PGA_2 , PGD_2 , PGE_2 and $\text{PGF}_{2\alpha}$ were gifts from Dr. J. Pike of the Upjohn Company, Kalamazoo, U.S.A. The pentane-acetone powder of ram seminal vesicle (RSV) was a gift from Dr. R.J. Flower of the Wellcome Research Laboratories, Beckenham, England.

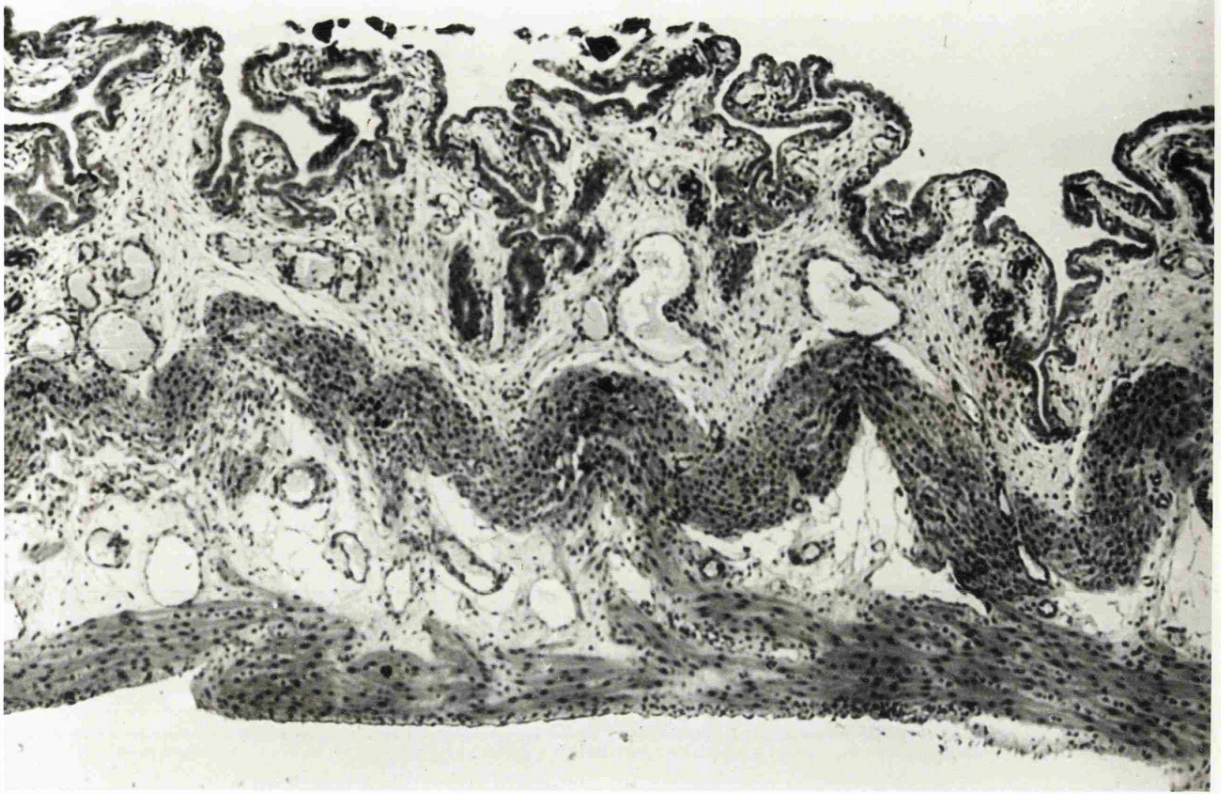
The following drugs which were used in this study were gifts from the manufacturing company:-

Drug	Company
Alcolofenac	Berk Pharmaceuticals Ltd.
Aspirin	Boots
Flurbiprofen	Boots
Metopirone	CIBA Laboratories
Fenclozic Acid	ICI
Indomethacin	Merck, Sharp & Dohme
Meclofenamate	Parke-Davis
5,8,11,14,Eicosatetraynoic Acid (TYA)	Roche Ltd.
Tranylcypromine	Smith, Kline & French

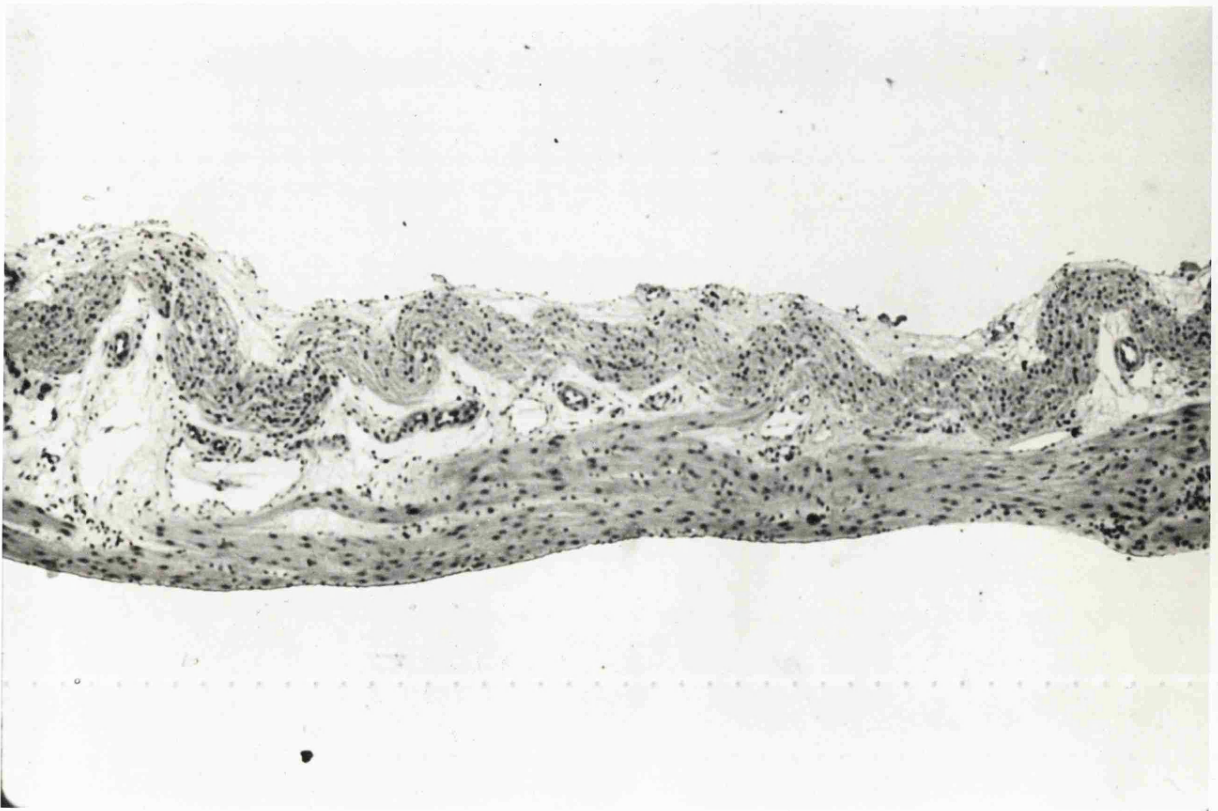
2.3 Methods

(i) Preparation of Decidual and Myometrial Microsomes: Female rats (CYF or Wistar strain) were killed on day 22 of pregnancy, expected day of delivery (day 1 of pregnancy was counted as the day of finding the cervical plug). The uterus was dissected out and its contents removed, it was then cleaned and weighed. In later experiments the myometrium lying under the placentae (MUP) was dissected out. The decidual and myometrial tissues were separated by scraping the luminal surface with a microscope slide (see Fig. 5). The myometrium was reweighed and the decidual weight calculated from the difference. An average tissue distribution would be as follows (Average of 15 uteri); average uterine weight 3.541 g, average myometrial weight 2.826 g, average decidual weight 0.141 g, average MUP weight 0.574 g. A 20% (w/v) homogenate of each fraction was then prepared in ice-cold buffer (as given in Section 2.1) using an Ultra Turrax homogeniser (full speed for 15 seconds). The homogenates were then centrifuged at 3000g for 30 minutes to prepare a cell free supernatant. The microsomes were obtained from this by further centrifugation at

Figure 5. The separation of decidua and myometrium. The top photograph shows longitudinal section through a rat uterus on day 22 of pregnancy. The upper decidual layer can be clearly distinguished from the lower myometrial layer, itself divided into circular and longitudinal muscle layers. The bottom photograph shows the same uterus after the decidua has been removed by scraping with a microscope slide. It can be seen that this technique removes the vast majority of the decidua without contaminating it with myometrium.



Uterus Before Scraping



Uterus After Scraping

100,000 g for 60 minutes. A typical yield of microsomes for decidual and myometrial tissue would be 2.45 mg and 3.31 mg per gramme of uterine tissue (-MUP) respectively.

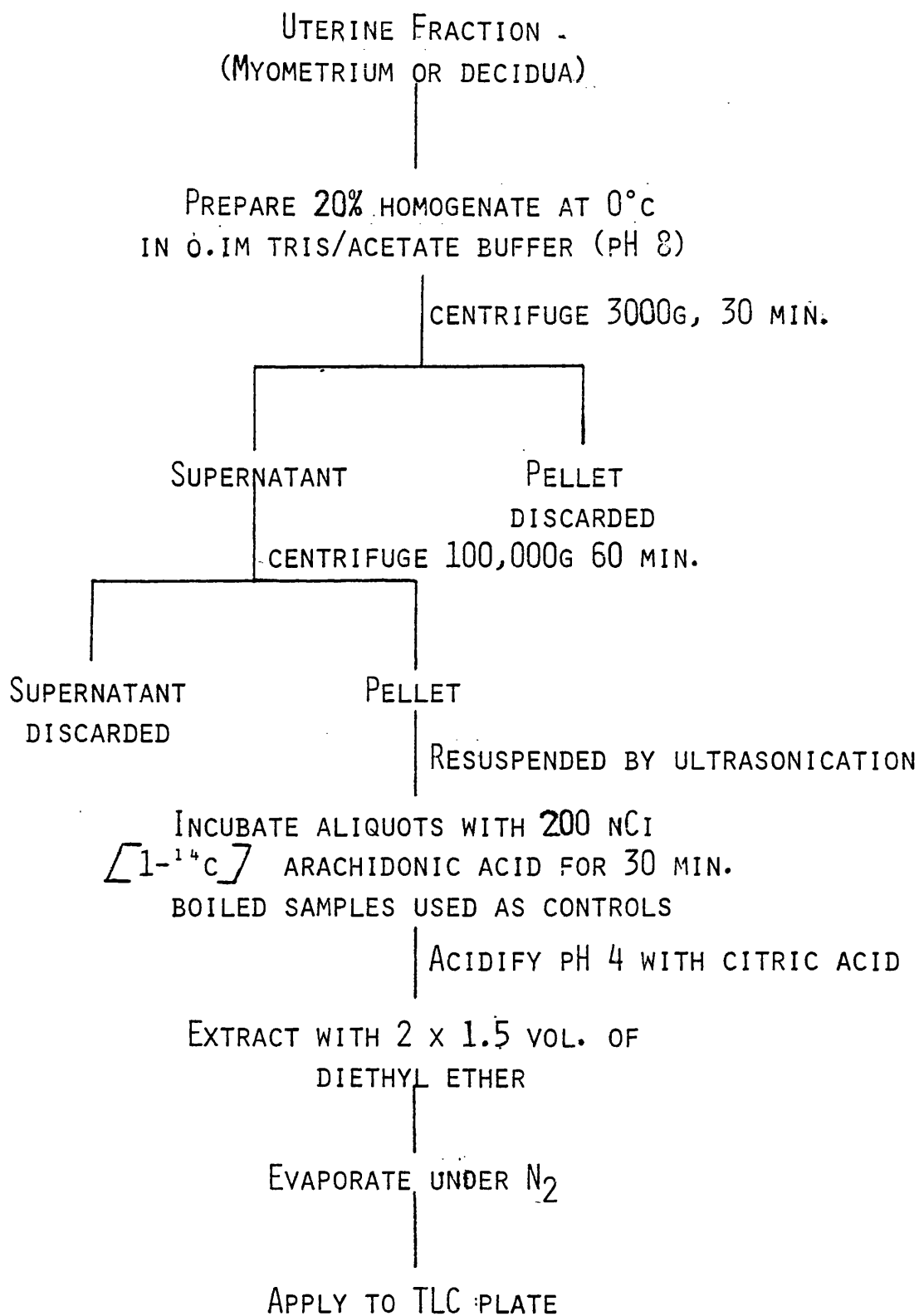
(ii) Assay for PG/TX Synthetase and Extraction of Products: The microsomal pellet obtained from the above procedure was then resuspended in a fresh volume of buffer by ultrasonication (20KHz, 150W for 15 seconds) to give a protein concentration of 1-2 mg/ml. Aliquots of the suspension were incubated, normally for 30 minutes with 1 ml of buffer containing arachidonic acid (99 nmoles) and 200 nCi of $[1-^{14}\text{C}]$ -arachidonic acid to give a total substrate concentration of 51 μM . The reaction was terminated by 0.25 ml of citric acid (2M). Samples of the microsomal suspension boiled before incubation served as controls.

For incubation of microsomes with NSAIDs, aliquots of the drugs were added to the microsomal suspension to give the required concentration of drug and a volume of 1.9 ml. Each incubate was then preincubated for 5 minutes before addition of the AA substrate (in 0.1 ml).

The PG-like products and unconverted substrates were extracted from the acidified incubation mixture with diethyl ether (2x1.5 vol). This was reduced to dryness under nitrogen at 37°C. The extraction procedure was verified using radio-labelled $\text{PGF}_{2\alpha}$, PGE_1 and AA.

(iii) Separation and Quantitation of PG-like Products: The above residues were redissolved in 40 μl of chloroform/methanol solution (1:1) and applied to a thin layer chromatography (TLC) plate (silica gel G, 0.25 mm thick), accompanied by authentic PG markers and AA. The TLC plate was then developed to 15 cm in chloroform/

Figure 6. Radiometric assay for the isolation and identification of PGs synthesized by decidual and myometrial fractions of pregnant rat uteri.



methanol/acetic acid/water (90:9:1:0.65). The markers were located with iodine vapour and the plate was then scanned for radioactivity (using a Bertold thin-layer scanner model LB2723). The radioactive zones in each lane corresponding to authentic PGs were then scraped off the plate into scintillation counting vials and eluted with 1 ml of methanol. 10 ml of scintillant was added and the radioactivity in each sample estimated using a liquid scintillation counter (Phillips Liquid Scintillation Analyser, Model 01). Since the final mixture of scintillant was heterogeneous, a quench correction curve based on a channels ratio was used to programme the calculator board in the counter which calculated the absolute activity (dpm). From the counts obtained it was possible to calculate the product formation (in pmol/min) from the expression below:

Product formed pmol/min =

$$\frac{\% \text{ substrate converted} \times \text{substrate concentration (pmol)}}{100 \times \text{time of incubation (minutes)}}$$

(iv) Characterisation of Prostaglandins from the Microsomal

Synthetase: The prostaglandin-like products formed by incubation of microsomal PG synthetase with $[1-^{14}\text{C}]$ -AA were characterised by chromatographic techniques using authentic PG markers; chemical conversion, and for the products of the decidual microsomes gas chromatography/mass spectrometry.

(a) Chromatographic Techniques: All chromatographs were carried out in lagged tanks allowed to equilibrate for 30 minutes before use. All TLC plates were silica gel G, 0.25 mm thick unless stated otherwise authentic markers were run with extracts. The PG markers were normally visualised with iodine vapour. This was unsuitable for silver nitrate impregnated plates in that case the markers were visualised with phosphomolybdic acid in ethanol.

System 1. Chloroform/methanol/acetic acid/water (90:9:1:Q.65) (Pace-Asciak, Morawska and Wolfe, 1970). This system was regularly used throughout the study for the separation of PG-like products from the unconverted substrate and any hydroxy fatty acids formed. It was also capable of adequately separating PGE_2 from $\text{PGF}_{2\alpha}$.

System 2. Chloroform/methanol/acetic acid (80:10:10) (Cottee, Flower, Moncada, Salmon, and Vane, 1977), used in conjunction with TLC plates impregnated with silver nitrate by spraying with a 5% silver nitrate solution in ethanol/water (7:3).

System 3. The organic phase of ethyl acetate/iso-octane/acetic acid/water (11:5:2:10) (Cottee, Flower, Moncada, Salmon, and Vane, 1977). This system, with System 2 was used to separate PGE_2 and 6-oxo- $\text{PGF}_{1\alpha}$, which co-chromatograph in System 1.

System 4. Diethyl ether/methanol/acetic acid (90:1:2) (Nugteren and Hazelhof, 1973). This system was used to separate PGE_2 from PGD_2 , which are not adequately separated by System 1.

System 5. Benzene/dioxan/acetic acid (20:20:1) used in conjunction with 5% silver nitrated TLC plates (Green and Samuelsson, 1964). This was used after the chemical conversion of PGE_2 by sodium hydroxide and sodium borohydride to identify PGB_2 , $\text{PGF}_{2\alpha}$ and $\text{PGF}_{2\beta}$.

(b) Chemical Conversion of $[1-^{14}\text{C}]-\text{PGE}_2$: After the initial development in System 1 the radioactive zone corresponding to PGE_2 was scraped off the TLC plate into a sintered glass funnel and eluted with methanol. The filtrate was divided into

two parts and reduced to dryness under nitrogen at 37°C. To one portion 2 ml of sodium hydroxide (0.5M) was added, whirly-mixed and incubated for one hour at 37°C. The second portion was taken up in water (pH 8.0) and solid sodium borohydride (10 mg) added, whirlymixed and again incubated at 37°C for one hour. Both samples were then acidified to pH 2.5 with citric acid (2M) and extracted with diethyl ether (2 x 1.5 vol). After evaporation the extracts were applied to a TLC plate and developed in solvent System 5.

(c) Gas Chromatography/Mass Spectrometry: The actual gas chromatography/mass spectrometry (GC/MS) and derivatisation was carried out by Drs' Dawson, Boot, Cockerill, Mallen and Osborne at the Lily Research Centre, Windlesham, Surrey. A preparative synthesis using 30 mg of decidual microsomes was carried out using the method described above. After the extracts had been chromatographed on System 1 and scanned the zones of radioactivity were scraped off into separate sintered glass funnels and eluted with methanol. These samples were then sent to the Lily Research Centre. From each sample the methyl ester, methyl oxine and trimethyl silyl ether derivatives were prepared and the products identified by GC/MS. The products were also quantitated against derivatives of authentic $\text{PGF}_{2\alpha}$. The full experimental details have been published (Cockerill, Mallen, Osborne, Boot and Dawson, 1977).

(v) Preparation of $[1-^{14}\text{C}]$ -6-oxo-PGF_{1 α} : The GC/MS analysis identified 6-oxo-PGF_{1 α} in one of the extracts which chromatographed with PGE₂. Authentic $[1-^{14}\text{C}]$ -6-oxo-PGF_{1 α} was prepared from $[1-^{14}\text{C}]$ AA (Cottee, Flower, Moncada, Salmon and Vane, 1977). An acetone-pentane powder of ram seminal vesicles (10 mg) was incubated in 1 ml

of Tris (0.05M, pH 7.5) with 0.5 μ g of $[1-^{14}\text{C}]$ -AA (100 μ Ci) without cofactors at room temperature. The incubation mixture was then extracted with diethyl ether (2 & 3 vol). The extracts were dried under nitrogen and applied to a TLC plate developed in System 3. Three radioactive zones were found with R_f equivalent to authentic 6-oxo-PGF_{1 α} , (approximately 65%), PGE₂ (approximately 25%) and AA (approximately 6%). The zone with a R_f equivalent to authentic 6-oxo-PGF_{1 α} was eluted with methanol. A sample was then run on TLC System 2 and the R_f checked to see if it corresponded with the published R_f values for 6-oxo-PGF_{1 α} . The rest was stored in methanol at -20°C.

(vi) Preparation of an Acetone-Pentane Dry Powder of Uterine Microsomes: This method was derived from that of Wallach and Daniels, (1971). The decidua and myometrium were separated as before but in potassium chloride (0.154 M) and the temperature kept between 0-3°C. The two fractions were homogenised using an Ultra-Turrax (full speed 15 seconds), and then centrifuged at 3000 g for 10 minutes. The supernatant was then filtered off through gauze. Acetone was then added slowly until it reached a concentration of 30%. The resulting precipitate was recovered by centrifugation, 3000 g for 15 minutes.

The precipitate was blended with approximately 15 volumes of acetone (w/v). This mixture was filtered on a chilled Buchner funnel using Whatman No: 4 paper. After removal of most of the solvent a rubber dam was stretched over the top of the funnel and the precipitate pressed until no further solvent could be removed. The precipitate was then quickly resuspended in approximately 7.5 volumes of acetone (w/v) and filtered as before. The precipitate was again resuspended, but this time in 5 volumes of pentane (w/v). Most of the pentane was removed as before; the precipitate was transferred to a round-bottomed

flask and the remaining pentane removed by evaporation in vacuo. When dry the precipitate was transferred to a vacuum desiccator over phosphorus pentoxide.

(vii) Concentration of Hydroquinone Replacement Cofactors:

Adrenaline, noradrenaline, 5-hydroxytryptamine, histamine and L-tryptophan were used at a concentration of 0.5 mM, the same as that of hydroquinone. Haemoglobin, used in conjunction with L-tryptophan at a concentration of 0.75 μ M. L-ascorbic acid at a concentration of 1 mM was used both to protect adrenaline and noradrenaline from auto-oxidation and as a cofactor. This concentration was selected as that concentration of L-ascorbic acid which would prevent formation of adrenochrome from adrenaline for at least two hours.

(viii) Qualitative Determination of PG Synthesis using

Sodium Thiobarbiturate: This method was adapted from that of Flower, Cheung and Cushman (1973). 2-Thiobarbituric acid (0.144 g) was suspended in 10 ml of water and sodium carbonate added (0.083 g in 6.6 ml of water) to give a 1% solution (w/v) of sodium thiobarbiturate. The uterine or RSV microsomes were incubated normally, the reaction being terminated with the addition of 0.1 ml of 100% trichloroacetic acid (w/v) in 1N hydrochloric acid. 0.1 ml of sodium thiobarbiturate was then added and the mixture boiled for 20 minutes. The resulting red colouration was clarified by centrifugation (3000 g for 10 minutes) and aliquots read at 532 nm. Incubation tubes which did not contain any microsomal enzyme preparation served as controls. This assay was used solely to determine whether PG synthesis occurred or not and no attempt at quantitation was made.

(ix) Preparation of NSAID Solutions: Solutions of these drugs were always freshly prepared before use. A stock solution of each drug (10^{-2} or 10^{-3} M) in the normal tris buffer (pH 8.0) used for routine incubations, either with the aid of a small quantity of absolute ethanol (400 μ l in 25 ml) or by using sodium carbonate to make the sodium salt.

(x) Protein Estimation: All protein estimations were determined by the method of Lowrey, Rosenbrough, Far and Randell (1951). Bovine serum albumin was used as a standard.

CHAPTER THREE

RESULTS

3.1 Validation of Extraction Procedure

The extraction of substrate and products from the acidified incubation medium by diethyl ether was investigated. 200 nCi of $[1-^{14}\text{C}]$ -AA, $[5,6,8,11,12,14,15 \text{ (n)}-^3\text{H}]$ -PGF_{2α} and $[5,6, \text{(n)}-^3\text{H}]$ -PGE₁ were added to separate 2 ml aliquots of buffer as used for routine incubations. Then each aliquot was acidified and extracted with three portions of diethyl ether (1.5 vol). Each portion was separately evaporated under nitrogen in scintillation counting vials. Methanol (1 ml) was added and subsequently 10 ml of scintillant, the radioactivity was then estimated by scintillation counting. The percentage recovery was then calculated. Table 1 shows that for both the standard PGs and for AA more than 97% could be recovered in the first two extractions, the number of extractions employed routinely.

Table 1. Recovery of ^{14}C -AA, ^3H -PGE₁ and ^3H -PGF_{1α} from acidified suspensions of uterine microsomes by diethyl ether. Percent recovery as percentage of original counts added.

	AA	PGE ₁	PGF _{2α}
1st Extraction	92.2 ± 0.5	92.1 ± 0.3	84.0 ± 0.7
2nd Extraction	6.1 ± 0.2	7.0 ± 0.4	13.5 ± 0.6
3rd Extraction	0.7 ± 0.3	1.1 ± 0.2	2.6 ± 0.1

3.2 Metabolism of PGs by the Uterine Microsomal Suspension

The possibility that the microsomal suspensions may have contained PG metabolising enzymes was investigated by adding tritiated PGE₁ and PGF_{2α} in place of the $[1-^{14}\text{C}]$ -AA normally added. The microsomal suspensions was then incubated for 30 minutes at 37°C and

extracted as for AA incubates. TLC indicated that for the decidual microsomes 93% and 96% of the radioactivity recovered had Rf values corresponding to authentic PGE_1 and $\text{PGF}_{2\alpha}$ respectively, while for the myometrium 94% and 96% of the radioactivity recovered had Rf values corresponding to authentic PGE_1 and $\text{PGF}_{2\alpha}$. There were no other peaks of radioactivity seen with the extracts of the tritiated $\text{PGF}_{2\alpha}$ incubates. However, with the extracts of the tritiated PGE_1 a small peak (3%) with an Rf of 0.3 was seen. This same peak was also detected when samples of tritiated PGE_1 were applied directly to the TLC plate and it was concluded that there was an impurity present in the stock solution of tritiated PGE_1 .

Tritiated PGE_1 was used in preference to PGE_2 as the latter was not available with a sufficiently high specific activity.

3.3 Products Formed During Incubation of the Uterine Microsomes

The initial chromatograph of the incubation products using solvent system 1 (chloroform/methanol/acetic acid/water, 90:9:1:0.65) showed the decidual and myometrial microsomes produced dissimilar products. A representative scan of a TLC of the decidual and myometrial incubation products by a Bertold TLC scanner is shown in Fig. 8. The dark areas indicate zones of radioactivity, the difference in AA metabolism between decidual and myometrial microsomes is clearly visible. The radioactive zone with an Rf below that of authentic $\text{PGF}_{2\alpha}$ seen on the decidual scan was found to be non-prostanoid in nature by a subsequent GC/MS analysis. It could be found in both decidual and myometrial incubates, although it is not clear in the myometrial scan depicted. When the total product formation was estimated this zone of radioactivity, and that zone with an Rf corresponding to that of monohydroxy fatty acids (MHFA) were excluded.

Figure 7. The distribution of radioactivity after incubation of uterine microsomes with tritiated $\text{PGF}_{2\alpha}$ and PGE_1 . Plates run in solvent system 1 (chloroform/methanol/acetic acid/water, 90:9:1:0.65). The radioactivity in consecutive 1 cm zones was then estimated by liquid scintillation counting.

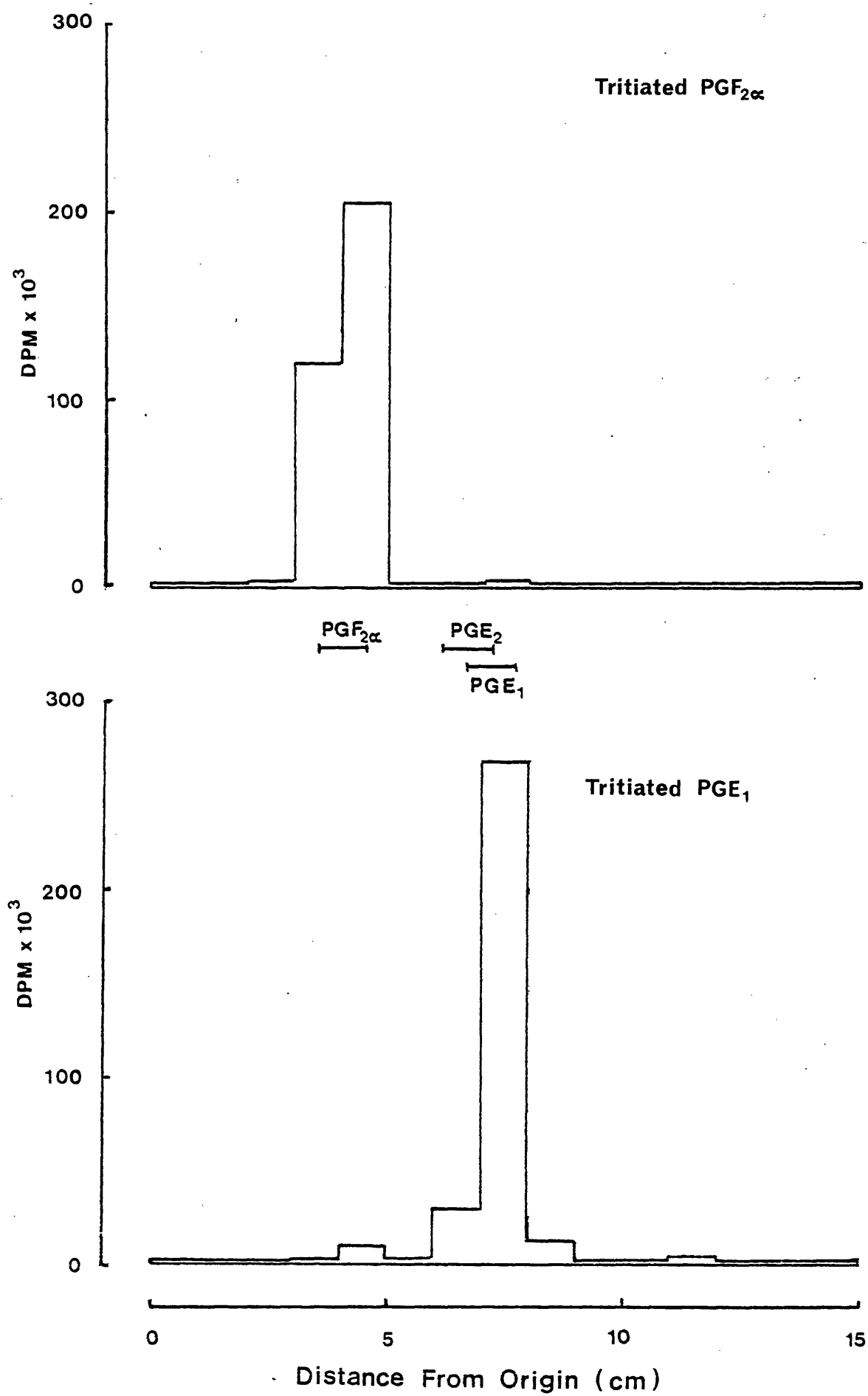
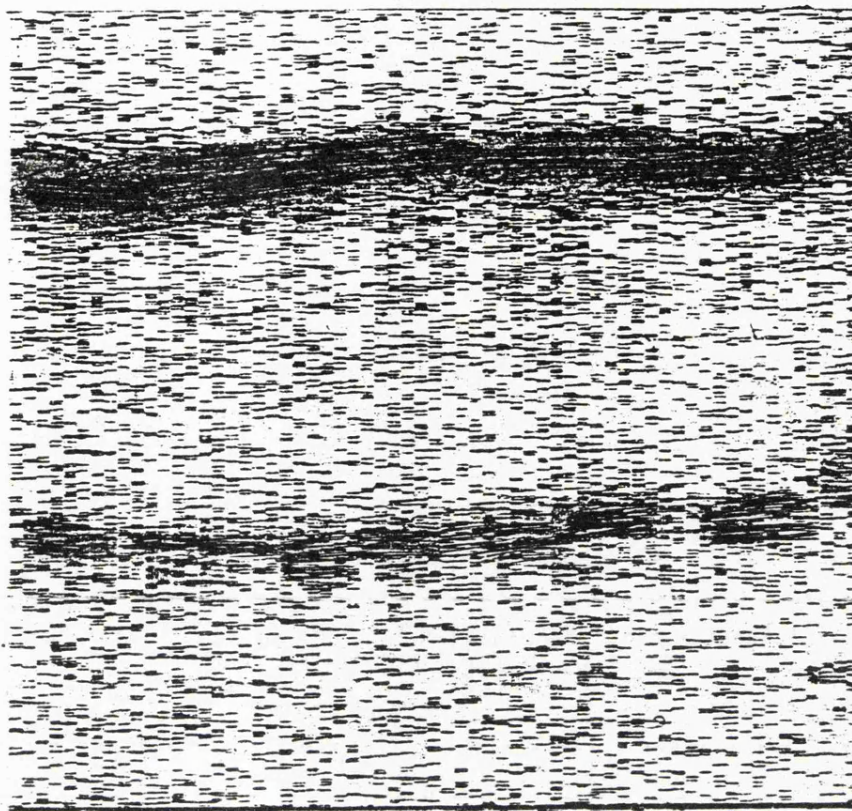


Figure 8. A scanning radiochromatogram of two TLC plates run in solvent system 1 (chloroform/methanol/acetic acid/water, 90:9:1:0.65). The upper plate contained extracts of myometrial incubates. The lower plate extracts of decidual incubates. The four product peaks for the decidual can be clearly seen, in contrast with the single peak of the myometrial. The radioactive zone just below the AA zone is due to monohydroxy fatty acids (MHFA). The zone below the authentic $\text{PGF}_{2\alpha}$ marker is an unknown, but GC/MS analysis indicated it was non-prostanoid in nature. It was therefore excluded, along with MHFAs when total product formation was assessed.

SOLVENT
FRONT ➤

MHFA |



| AA

| PGE₂

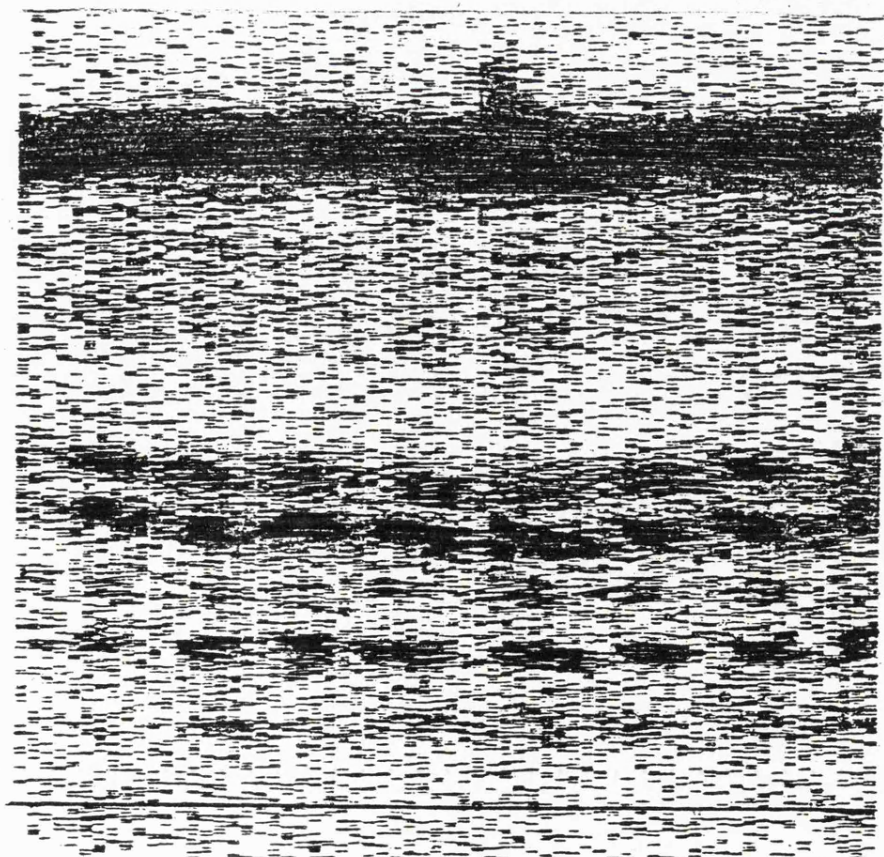
| PGF_{2α}

ORIGIN ➤

MYOMETRIUM

SOLVENT
FRONT ➤

MHFA |



| AA

| PGE₂

| PGF_{2α}

ORIGIN ➤

DECIDUA

Fig. 9 shows a histogram of the distribution of incubation products of decidua and myometrial microsomes, minus the boiled control values (to avoid a negative value for the unconverted AA that peak was omitted). The chromatogram of the extracts from the decidua incubation revealed six major peaks, one with an R_f of 0.93 corresponded to authentic AA (omitted in Fig. 9) while a second had an R_f similar to that expected of MHFAs. (The MHFA marker was prepared from AA using soybean lipoxidase). The four remaining peaks had R_f s of 0.25 (peak A) corresponding to authentic $\text{PGF}_{2\alpha}$, 0.33 (peak B), 0.43 (peak C) corresponding to authentic PGE_2 and 0.48 (peak D). In contrast the chromatogram of the extracts from the myometrial incubations showed only three peaks, again one with an R_f corresponding to authentic AA (omitted from Fig. 9), and a second with an R_f expected of MHFAs. The third peak has an R_f corresponding to that of authentic PGE_2 .

The zones of radioactivity corresponding to authentic PGE_2 from both decidua and myometrial incubations were scraped from the TLC plates and reacted with sodium hydroxide or sodium borohydride. Sodium hydroxide treatment produced a radiolabelled product with an R_f corresponding to PGB_2 when run in solvent system 5 (benzene/dioxan/acetic acid, 20:20:1, in conjunction with 5% silver nitrated plates). The sodium borohydride treatment converted the PGE-like incubation product to a compound with an R_f corresponding to that of authentic $\text{PGF}_{2\alpha}$. In retrospect when it was realised that 6-oxo- $\text{PGF}_{1\alpha}$ was present in the zones used, these reactions were repeated and the products run in solvent system 3 (the organic phase of ethyl acetate/iso-octane/acetic acid/water, 11:5:2:10) which was designed to separate 6-oxo- $\text{PGF}_{1\alpha}$ from other prostaglandins. This revealed that 6-oxo- $\text{PGF}_{1\alpha}$ was unaffected by sodium hydroxide treatment, but was converted by

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Figure 9. Metabolism of $[1-^{14}\text{C}]$ -AA by decidua and myometrial microsomes. The histograms show the distribution of radiolabel after the products of incubation were extracted from acidified solution with ether. After evaporation the products were run in a solvent system of chloroform/methanol/acetic acid/water (90:9:1:0.65). The radioactivity in consecutive 0.5 cm zones of the sample and a boiled control were estimated. The histogram shows net d.p.m. i.e. test minus boiled control values. The broken line at the solvent front indicates far more unconverted AA in the boiled control sample, as this would have given a negative value a dotted line has been used.

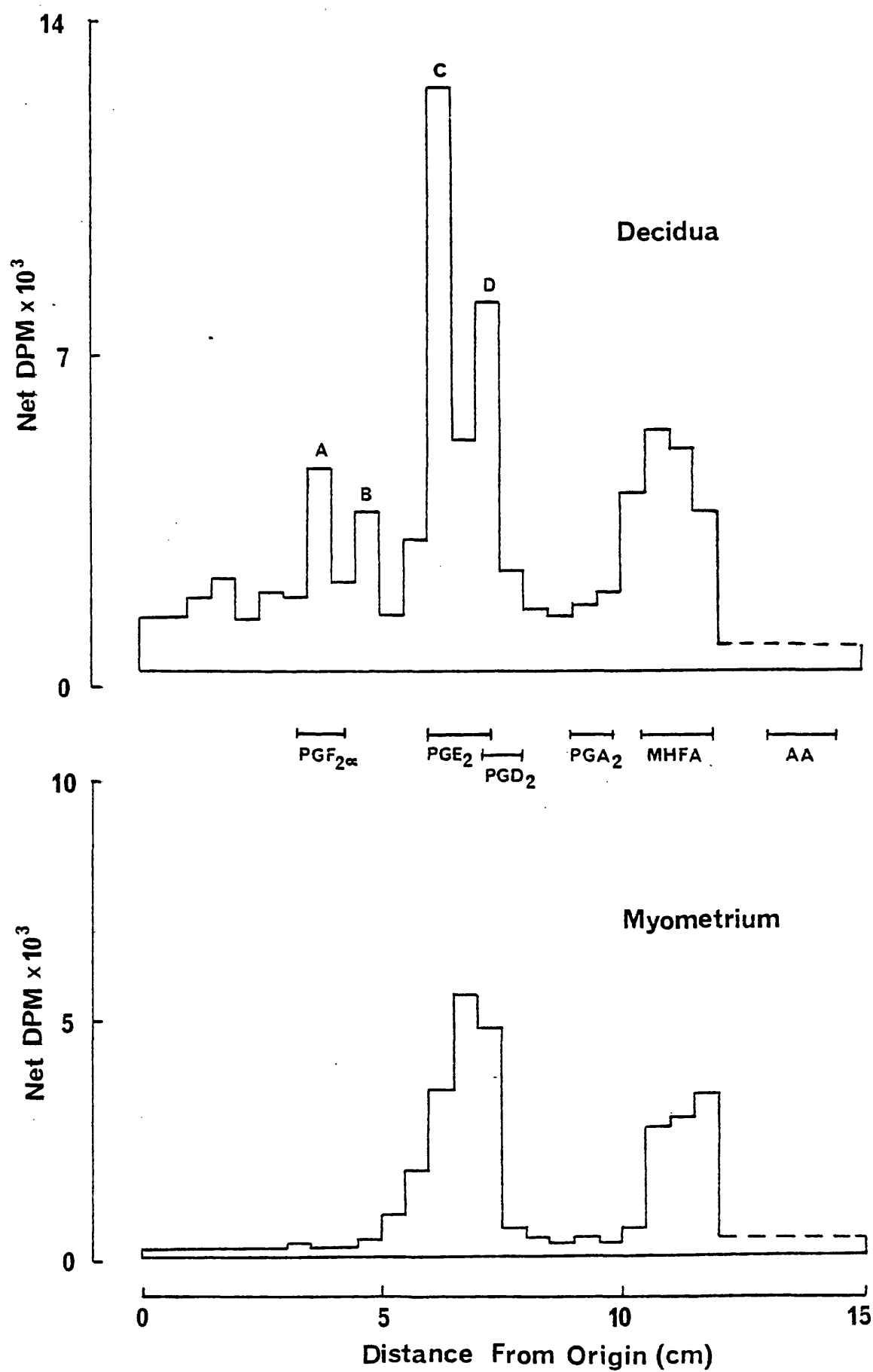
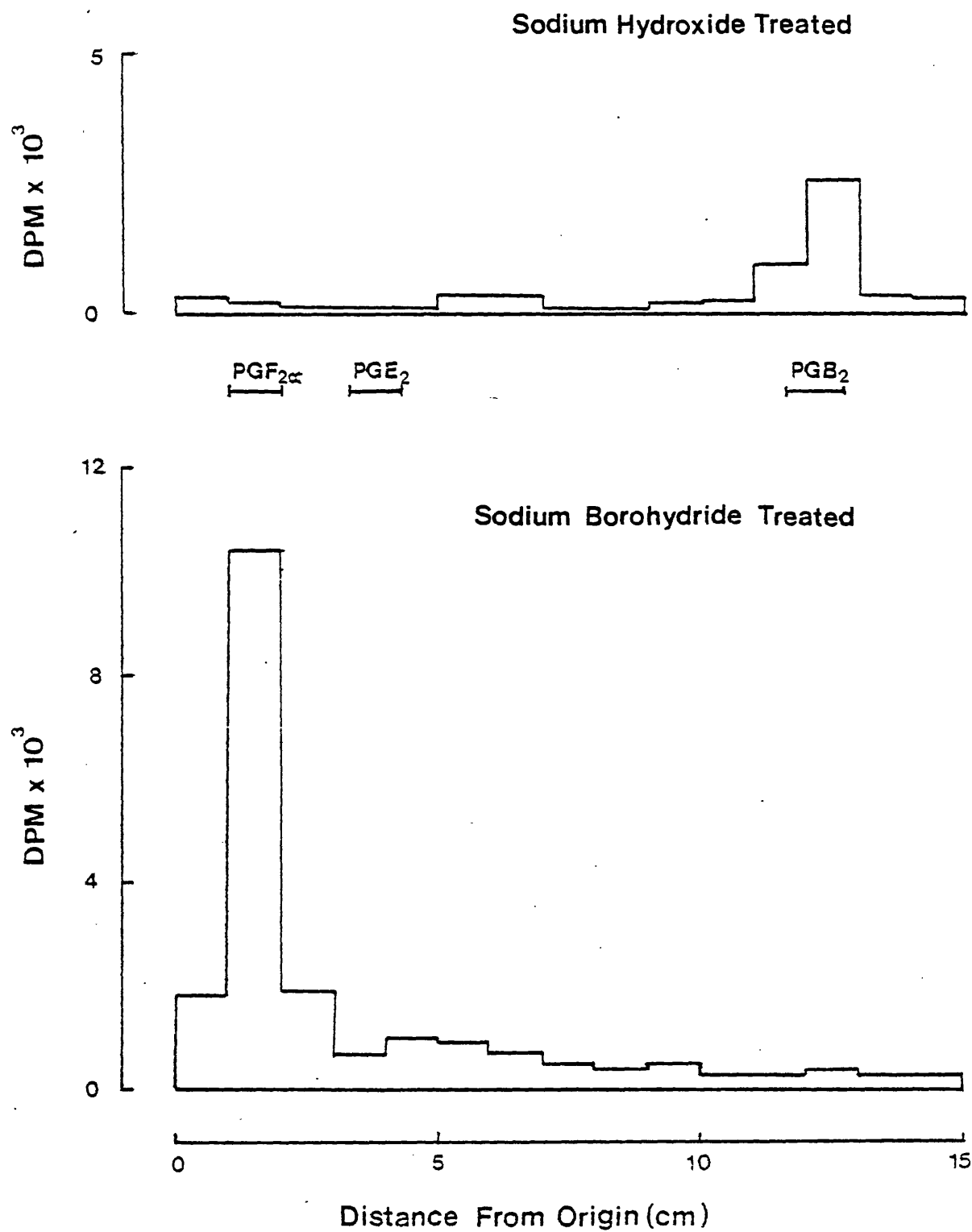


Figure 10. Distribution of radioactivity seen after treatment of the PGE zone with sodium hydroxide or sodium borohydride. the products formed from these chemical reactions were extracted and developed in solvent system 5 (benzene/dioxane/acetic acid, 20:20:1 on 5% silver nitrated plates). The plate was then divided into successive 1 cm zones and the radioactivity in each zone evaluated.



sodium borohydride to a compound which had an Rf similar to that of $\text{PGF}_{2\alpha}$.

GC/MS analysis of the decidual product peaks A,B,C and D revealed that peak A was $\text{PGF}_{2\alpha}$, peak B was TxB_2 , peak C a mixture of PGE_2 and 6-oxo- $\text{PGF}_{1\alpha}$ and peak D was PGE_2 . It was decided to supplement the GC/MS data concerning peak C by assessing the relative proportions of the PGs within this peak and to carry out a further investigation on the myometrial peak of radioactivity which like peak C of decidual microsomes also had an Rf value corresponding to authentic PGE_2 . For this purpose two TLC solvent systems developed by Cottee, Flower, Moncada, Salmon and Vane (1977) were employed. For the sake of brevity these systems have been termed system 2 (chloroform/methanol/acetic acid, 80:10:10, using 5% silver nitrated plates) and system 3 (the organic phase of ethyl acetate/iso-octane/water/acetic acid 11:5:10:2). Extracts from the incubation media were first separated in solvent system 1. The peaks of radioactivity corresponding to PGE_2 for both myometrial and decidual microsomes were then scraped off, the radioactivity eluted and rechromatographed in one or both of the systems described. The distribution of radioactivity seen using system 2 is shown in figure 11. For the decidual peak C this solvent system confirmed the presence of 6-oxo- $\text{PGF}_{1\alpha}$ ($36.1 \pm 2.5\%$ of the radioactivity had a mobility corresponding to authentic 6-oxo- $\text{PGF}_{1\alpha}$, $n = 13$) although the majority of the radioactivity still had an Rf corresponding to PGE_2 ($53.9 \pm 4.0\%$, $n = 13$). In contrast this TLC system showed the majority of the radioactivity in the myometrial peak to have an Rf similar to authentic 6-oxo- $\text{PGF}_{1\alpha}$ ($76.6 \pm 2.0\%$, $n = 13$) with only a small quantity having an Rf corresponding to authentic PGE_2 ($11.6 \pm 1.8\%$, $n = 13$). Similar results were obtained using solvent system 3, the distribution of radioactivity is shown in Fig. 12.

Figure 11. Thin-layer chromatogram of decidual peak C and myometrial products peak from TLC system 1 rechromatographed in solvent system 2 (chloroform/methanol/acetic acid, 80:10:10, using 5% silver nitrated plates). The plate was then partitioned into consecutive 1 cm zones and the radioactivity in each zone estimated. It can be seen that whereas in the decidual peak C the majority of the radioactivity has an Rf corresponding to authentic PGE_2 in the myometrial product peak the majority of the radioactivity has an Rf corresponding to authentic 6-oxo-PGF_{1 α} .

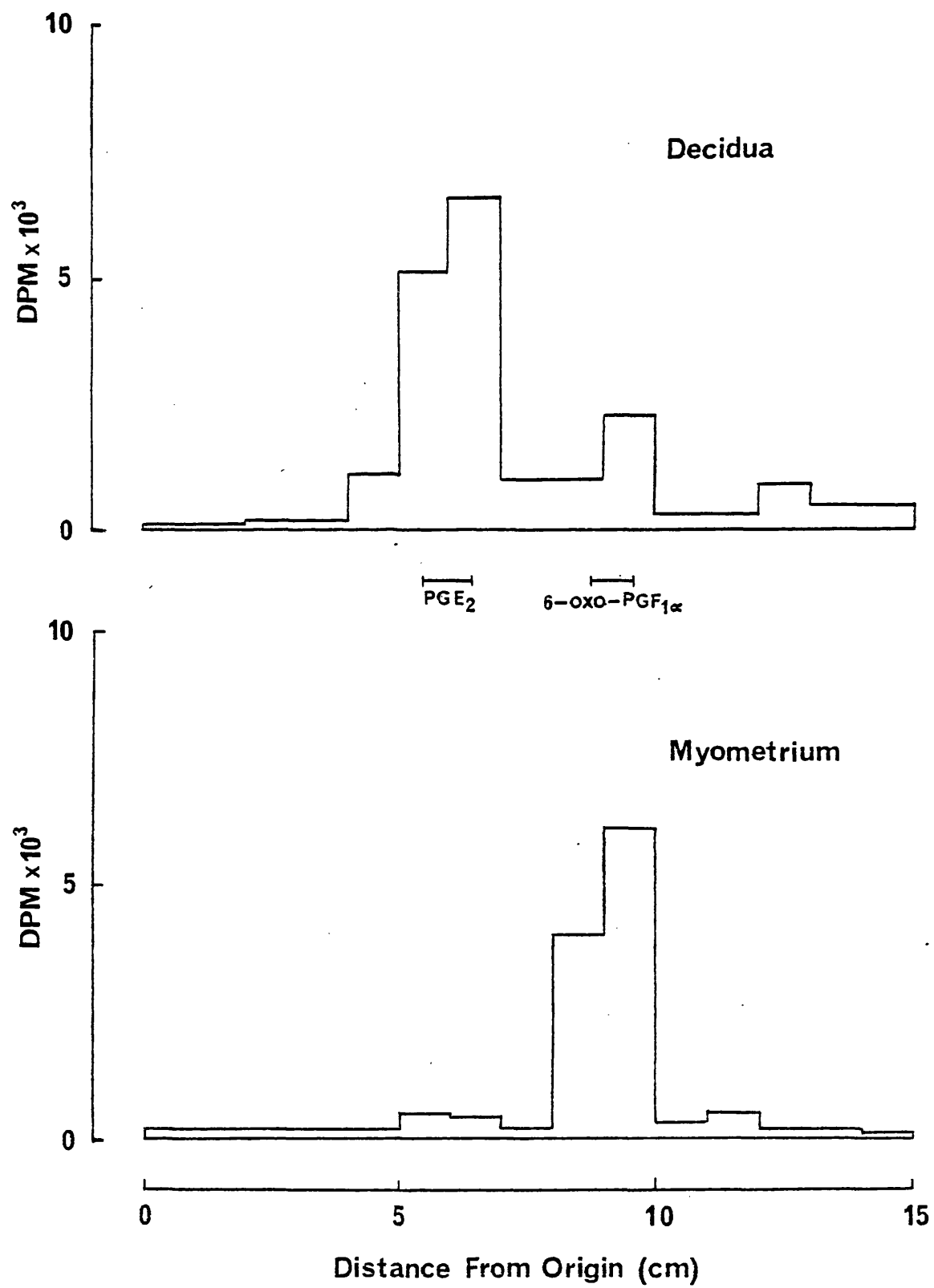
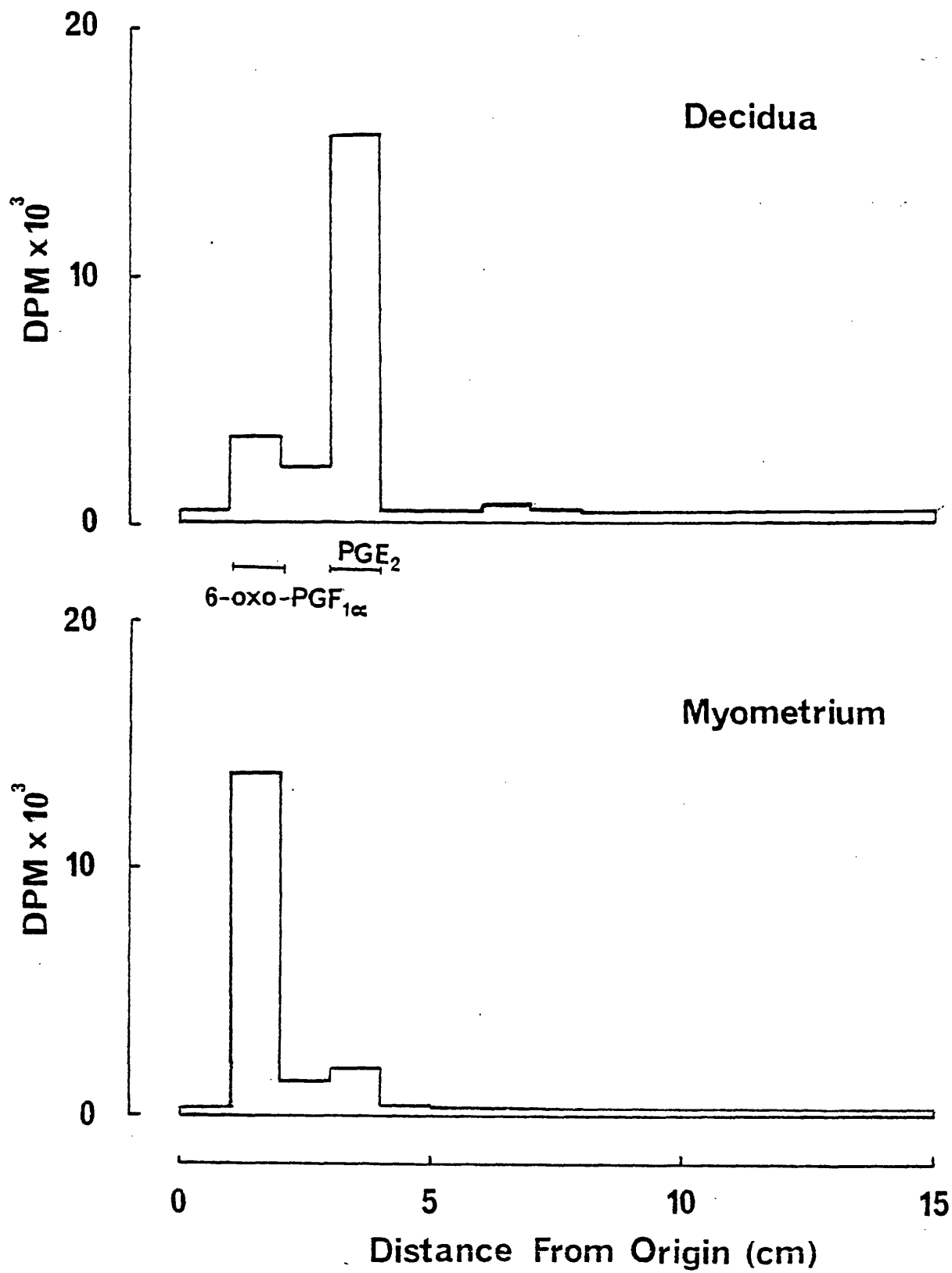


Figure 12. Spectrum of radioactivity after rechromatographing the peaks from decidual and myometrial incubations corresponding to PGE_2 in TLC system 1. The peaks of radioactivity were rerun in a solvent system comprising the organic phase of ethyl acetate/iso-octane/water/acetic acid, 11:5:10:2. (solvent system 3). The plate was divided into successive 1 cm zones and the radioactivity estimated. The major product in the decidual peak had an R_f corresponding to authentic PGE_2 , while in contrast the major product in the myometrial peak had an R_f corresponding to 6-oxo-PGF_{1 α} .



This confirmed that the radioactivity in the myometrial peak consisted predominantly of material with an Rf corresponding to authentic 6-oxo-PGF_{1α} ($80.4 \pm 2.9\%$, $n = 13$) again with a small percentage with an Rf corresponding to authentic PGE₂ ($10.5 \pm 1.2\%$, $n = 13$). The finding for the decidual peak C also agreed with that found in the previous TLC system $50.8 \pm 1.3\%$ ($n = 14$) of the radioactivity had an Rf corresponding to authentic PGE₂ and $36.8 \pm 3.5\%$ ($n = 14$) had an Rf corresponding to authentic 6-oxo-PGF_{1α}.

Decidual peak D was also subject to further investigation. The decidual incubation products were again separated in solvent system 1, peak D was then scraped off and the radioactivity eluted and rechromatographed in a solvent system developed by Nugteren and Hazelhof (1973) (diethyl ether/methanol/acetic acid, 90:1:2) and for brevity termed solvent system 4. The distribution of radioactivity seen using this system is shown in Fig. 13. It revealed that $45.6 \pm 1.6\%$ ($n = 27$) of the radioactivity present in the decidual peak D had no Rf corresponding to authentic PGE₂, while $60.9 \pm 1.1\%$ ($n = 27$) had an Rf corresponding to authentic PGD₂.

The proportions of each product synthesized by the decidual and myometrial microsomes is shown in Table 2, estimated by both GC/MS and TLC techniques for the decidua. It must be noted that there was an inevitable loss of radioactivity when products were separated in two TLC systems, since the products were expressed as a percentage of the total counts on the plate. However, this loss was relatively small and consistent, being for peak C $13.4 \pm 1.2\%$ ($n = 27$), for peak D $13.2 \pm 1.3\%$ ($n = 26$) and when estimating the overall composition of products formed for decidual and myometrial microsomes was $7.2 \pm 1.1\%$ ($n = 27$) and 11.5 ± 0.8 ($n = 26$).

Figure 13. Distribution of radioactivity obtained when peak D of decidual products in system 1 was rechromatographed in solvent system 4 (diethyl ether/methanol/acetic acid, 90:1:2). The radioactivity in consecutive 1 cm zones was then found. This revealed that the products associated with decidual peak D were approximately equally divided into material with Rfs corresponding to PGE_2 and authentic PGD_2 .

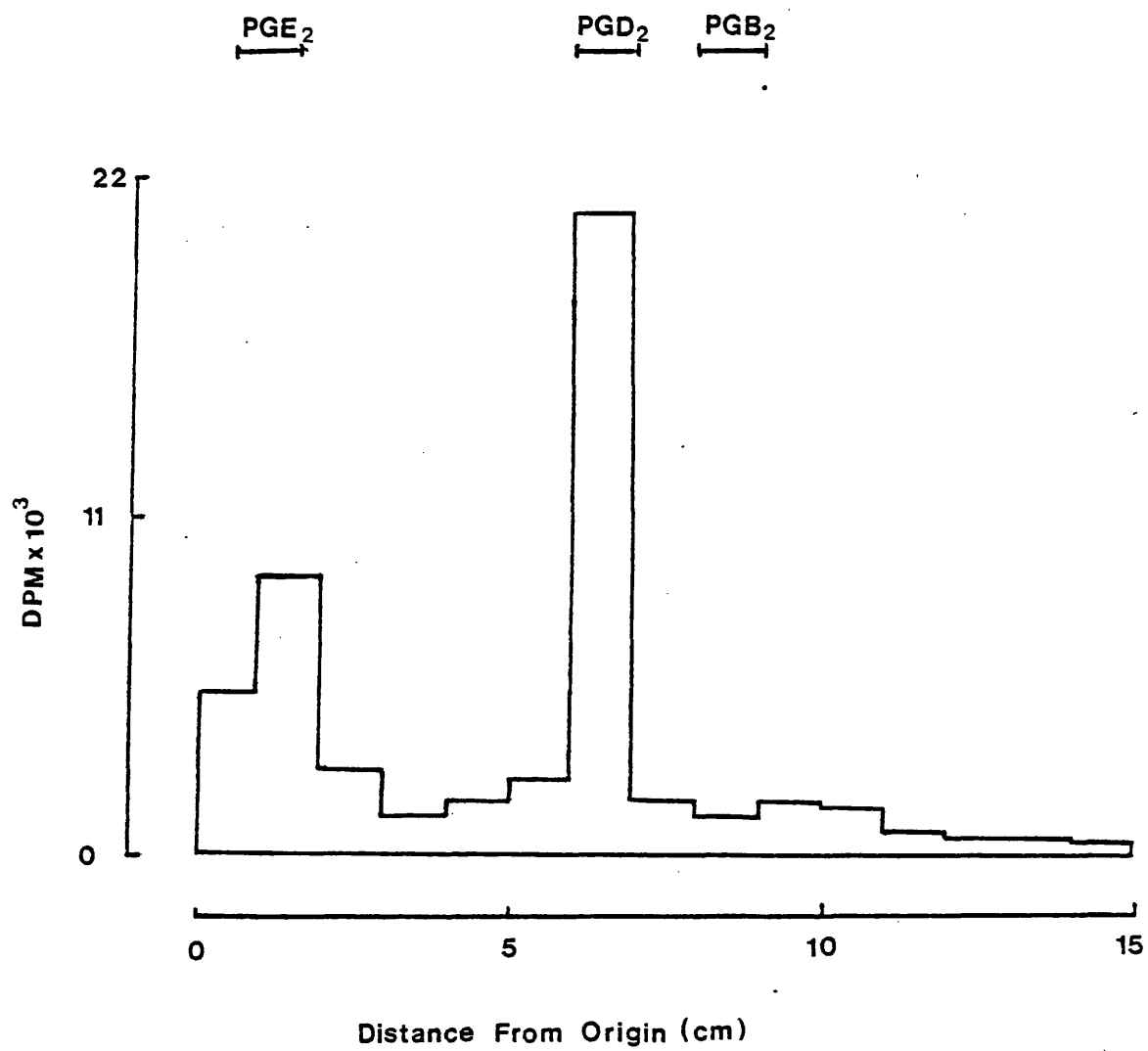


Table 2 Proportions of each PG-like product synthesized during the incubation of decidual and myometrial microsomal suspensions with $[1-^{14}\text{C}]$ -AA (expressed as % of the $[1-^{14}\text{C}]$ -AA converted to PG-like products).

Tissue	% Composition of PG-like Products				
	PGF _{2α}	TxB ₂	PGE ₂	6-oxo-PGF _{1α}	PGD ₂
Decidua estimated by TLC	17.8±1.1	12.8±2.8	37.4±3.0	15.0±1.5	10.7±1.3
Decidua estimated by GC/MS (1 expt. only)	11	3	74	12	0
Myometrium estimated by TLC	0	0	10.9±1.0	78.5±1.8	0

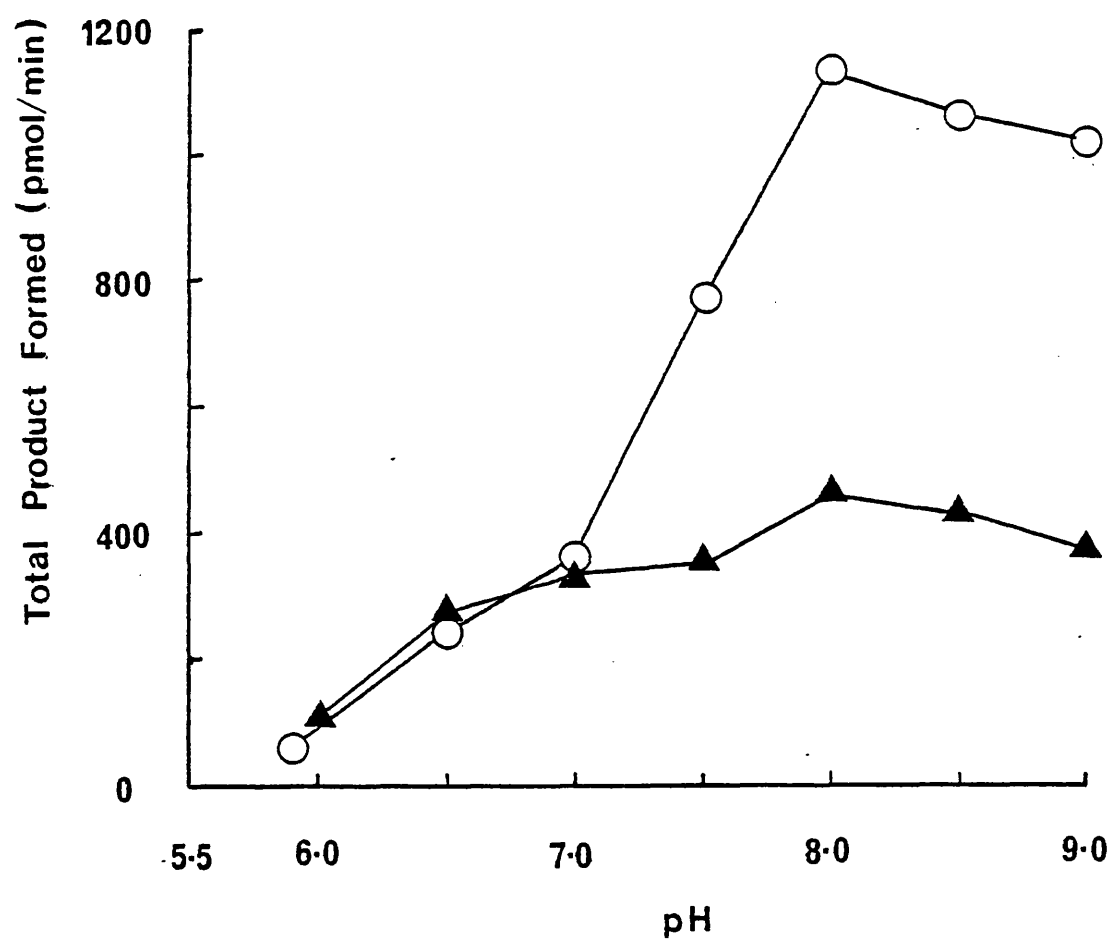
The overall synthesis of PG-like products from $[1-^{14}\text{C}]$ -AA, excluding MHFAs was, for the decidual microsomes $27.4 \pm 1.4\%$ or 28.0 ± 1.4 nmol ($n = 65$), over 30 minutes. While that for the myometrial microsomes was $10.2 \pm 0.7\%$ or 10.5 ± 0.7 nmol ($n = 65$) over 30 minutes, a much lower conversion than seen with decidual microsomes.

3.4 Incubation Conditions

(i) pH:

The effects of pH on conversion of substrate to products was examined over the range pH 6.0-9.0, for both decidual and myometrial microsomes. The optimal pH in both cases was found to be pH 8.0 (see Fig. 14). Either an increase or a decrease in pH from this optima adversely affected substrate metabolism. The PG synthetase complex was more tolerant to an increase in pH (with a reduction of

Figure 14. The effect of alteration of pH on rate of total PG-like product formation by decidual (O-O) and myometrial (▲-▲) microsomes. All other incubation conditions were kept constant. A pH optima of 8.0 was noted for both preparations.



approximately 6%, in synthesis for both decidual and myometrial microsomes at pH 8.5). A decrease in pH of half a unit produced a much larger inhibition of product synthesis (at pH 7.5 a 30% reduction in synthesis for both microsomal preparations was noted).

(ii) Incubation Time

Typical time courses of AA metabolism to PG-like products are shown in Fig. 15. It can be seen that with decidual incubations^b nearly half the products are synthesized in the first 5 minutes. Substrate conversion continued at a steady rate for the next 25 mins. doubling the conversion seen at 5 minutes. This was paralleled in the myometrial microsomal preparation to a lesser degree. To obtain maximum PG production particularly for the myometrial microsomes where AA metabolism appears intrinsically low an incubation time of 30 minutes was selected for routine incubations.

(iii) Protein Concentration

There did not appear to be a linear relationship between the protein concentration of the microsomal suspension and substrate conversion for either of the two uterine fractions over the range, 0.7-5.7 mg for decidual microsomes and 0.9-8.1 mg for myometrial microsomes (see Fig. 16). Because there was no linear relationship, expression of product formation in terms of microsomal protein concentration was avoided. As far as possible within experiments, protein concentration was kept constant. In different experiments protein concentrations varied between approximately 0.75-2 mg per incubation.

(iv) Substrate Concentration

The effect of varying the substrate concentration upon product formation is shown in Fig. 17. For the decidual microsomes there was a linear increase in product formation between 10 μ M-130 μ M.

Figure 15. Variation in AA conversion with time in decidual (O-O) and myometrial (▲-▲) incubations. It can be seen that nearly half of the decidual PG synthesis occurs in the first 5 minutes, conversion continued at a steady rate for the next 25 minutes, doubling that seen at 5 minutes. This was paralleled in the myometrial microsomal incubations, which had a lower synthetic capacity than the decidual microsomes.

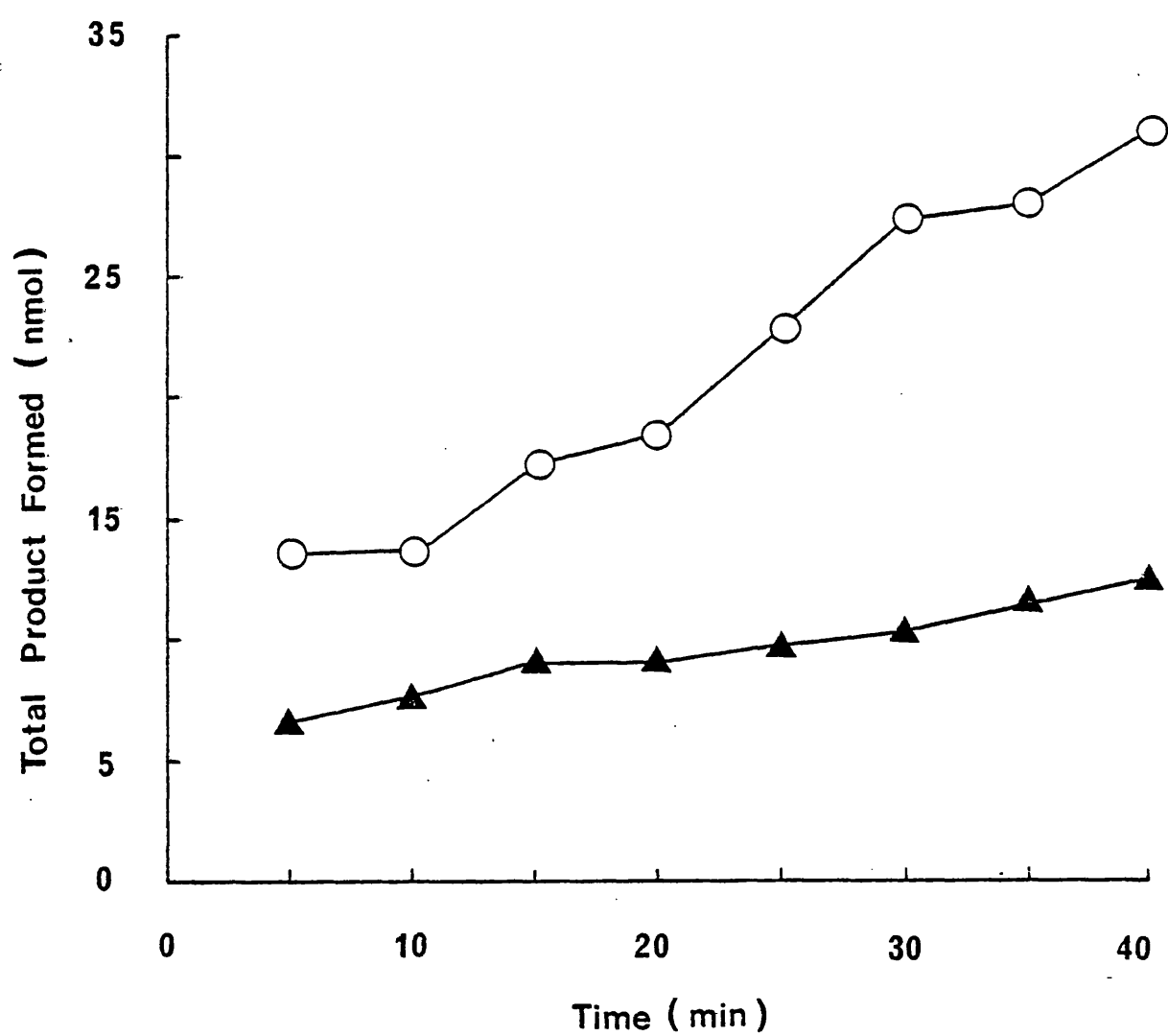
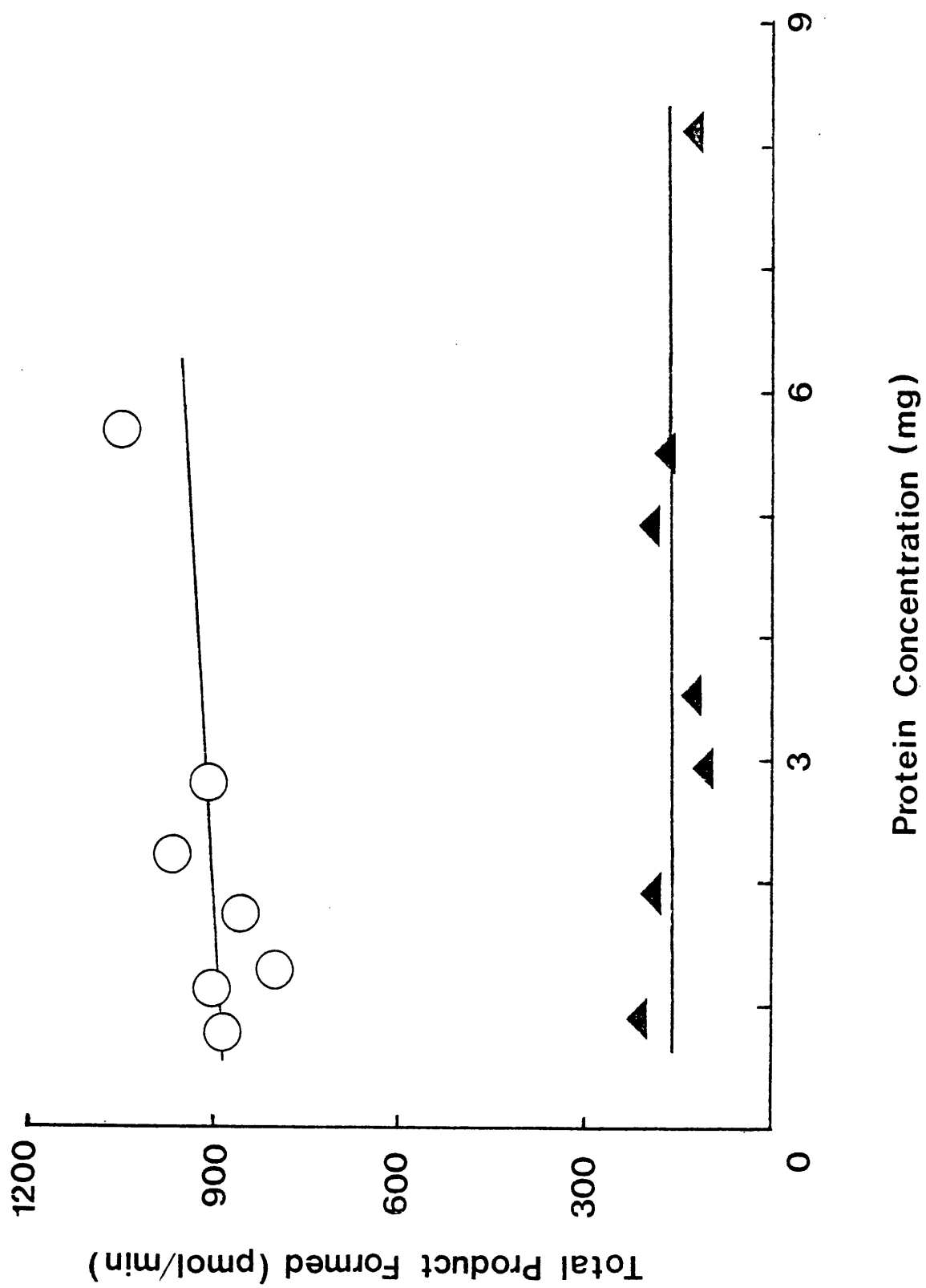


Figure 16. Effect of protein concentration on rate of production

formation. With both decidua (O-O) and myometrial (▲-▲) microsomes product formation appears independent of microsomal protein concentration.



However, above this value there was a sharp and progressive decrease (a 36% reduction at 250 μ M). In comparison product formation by myometrial microsomes was at a much lower level over the range of substrate concentrations studied. It appeared to be maximal at 51 μ M of AA. Although much less pronounced, product formation by myometrial microsomes also declined after peaking at 51 μ M with increasing AA concentrations. To achieve maximum conversion of the radiolabelled AA a substrate concentration of 51 μ M was used routinely.

(v) Cofactors

Microsomal preparations have been found to give better yields of products if the incubation medium contains exogenous cofactors. The buffer used in this study contained hydroquinone, GSH and L-cysteine for this purpose.

(a) Hydroquinone: The initial experiments had been designed so that the microsomes were resuspended in hydroquinone-free buffer, after which aliquots were adjusted to the required hydroquinone concentration. However, it was found that if this procedure was followed then the enzyme preparations from both decidual and myometrial fractions were inactive. To obtain an active enzyme preparation it was critical for the hydroquinone to be present while the microsomes were resuspended.

Fig. 18 shows that with decidual microsomes maximal product conversion occurred at 0.5mM of hydroquinone. After product formation was adversely affected so that at 0.75mM there was a 45% decrease in product formation. With myometrial microsomes variation in hydroquinone concentration had much less effect, giving an optimal range between 0.25-0.75mM. For routine

Figure 17 The effect of increasing substrate concentration on PG-like product synthesis by decidual (O-O) and myometrial (Δ - Δ) microsomes. There is a marked substrate inhibition with the decidual microsomes above 130 μ M AA. Substrate inhibition also occurs with the myometrial microsomes but at a lower concentration of AA (51 μ M).

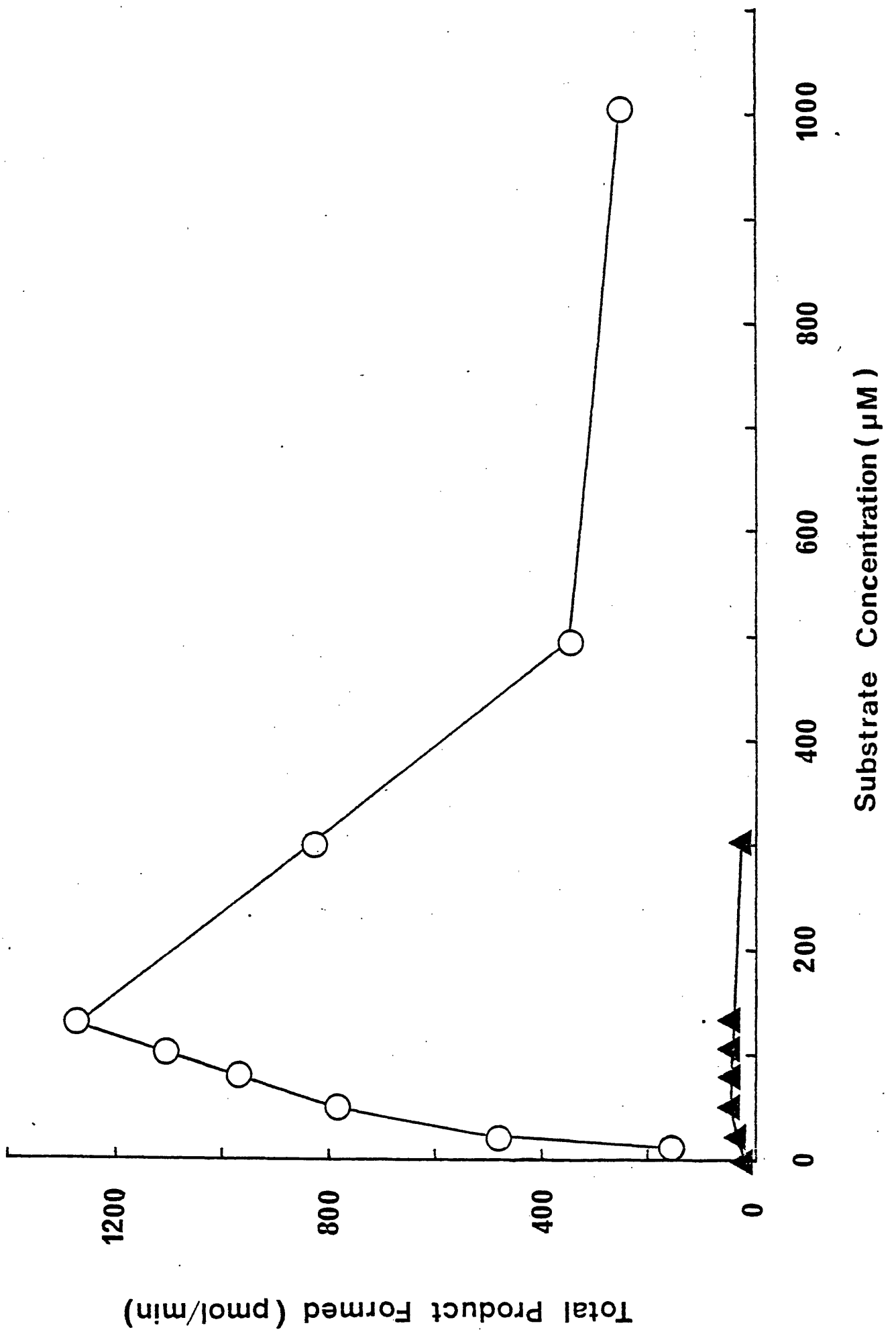
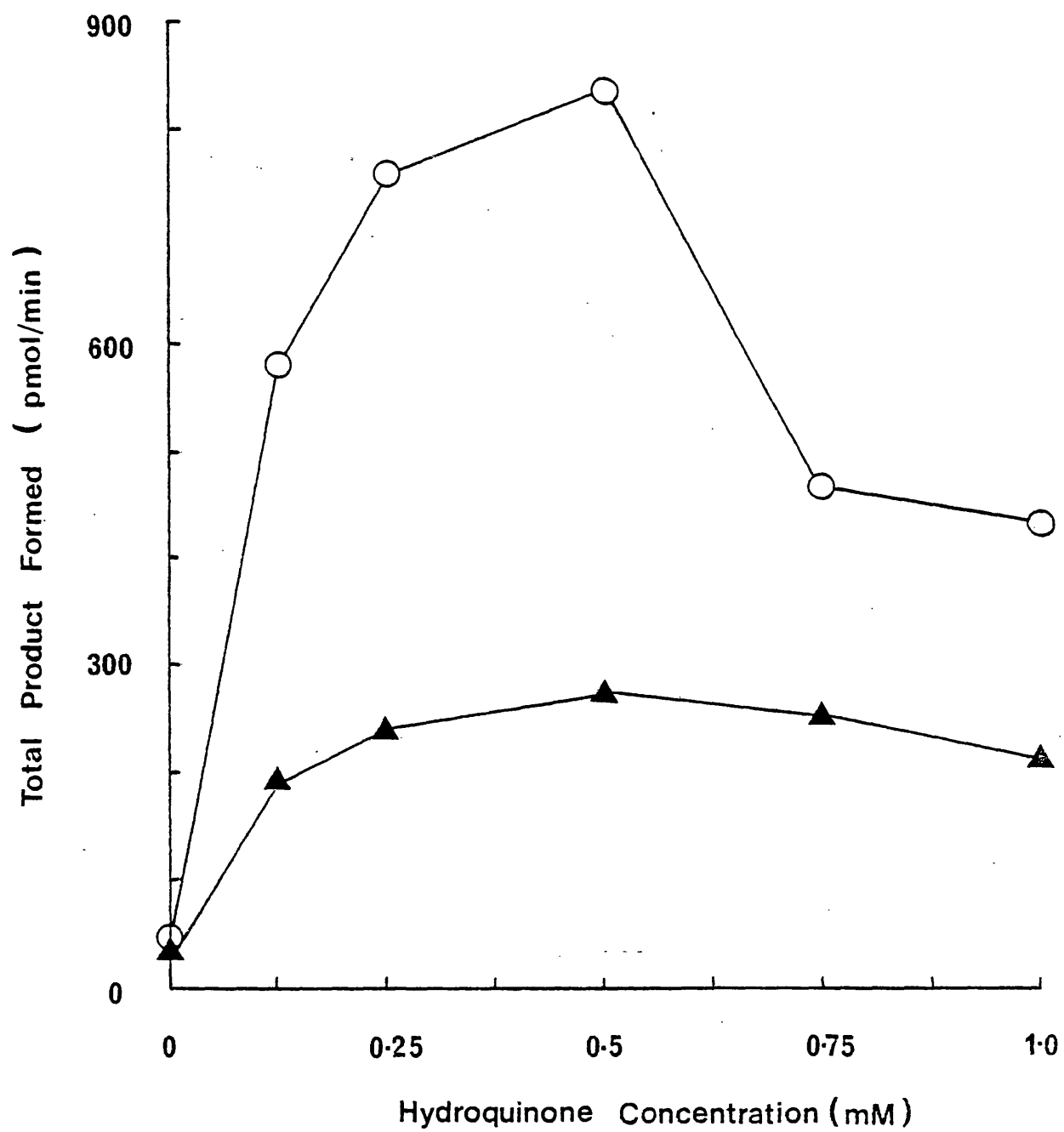


Figure 18. The effect of increasing hydroquinone concentrations on formation of PG-like products by decidual (O-O) and myometrial (▲-▲) microsomes. The optimal concentration for both tissue fractions was 0.5mM of hydroquinone. Above this value there was considerable inhibition with decidual microsomes. The myometrial microsomes were also inhibited by concentrations of hydroquinone greater than 0.5mM, but much less drastically than decidual microsomes.



assays a concentration of 0.5mM of hydroquinone was used for both tissue fractions.

(b) GSH: The optimal concentration of GSH for maximum product formation by both microsomal preparations was 2mM. The absence of GSH from the incubation mixture did not prevent biosynthesis taking place, but reduced it by approximately 50% for both decidua and myometrial microsomes. Increasing the concentration of GSH above 2mM caused a decrease in product formation, reducing AA metabolism by decidua microsomes by 35% at 3mM, but myometrial synthesis was reduced by only 11% at 3mM GSH. A concentration of 2mM GSH was used for both tissue fractions in routine assays.

(c) L-Cysteine: For the decidua microsomes there was a dramatic rise in product biosynthesis with the increase in L-cysteine concentration up to 1mM, which was optimal. Biosynthesis increased by 60% between 0-1mM L-cysteine. At 1.5mM there was a 30% decrease in synthesis of products. The myometrial microsomes were much less affected by alterations in L-cysteine concentrations, having an optimum range between 0.5-1.5mM. This increased the product formation by 17% at maximum over incubations without L-cysteine. In routine assays a concentration of 1mM L-cysteine was used for both decidua and myometrial microsomes.

(d) The effect of SH containing Cofactors on Products formed:
Owing to the possibility that the SH containing cofactors may have preferentially influenced the formation of one of the products synthesized by the microsomes it was decided to examine microsomal preparations of both tissue fractions in the presence

Figure 19. The effect of increasing GSH concentrations on PG-like product formation by decidual (O-O) and myometrial (▲-▲) microsomes. The optimal concentration of GSH for decidual microsomes was 2mM, with marked reduction in synthesis of products if this concentration of GSH is increased or decreased. For myometrial microsomes the optimum GSH concentration was again 2mM. Concentrations of GSH above or below this did reduce product synthesis but not as sharply as with decidual microsomes.

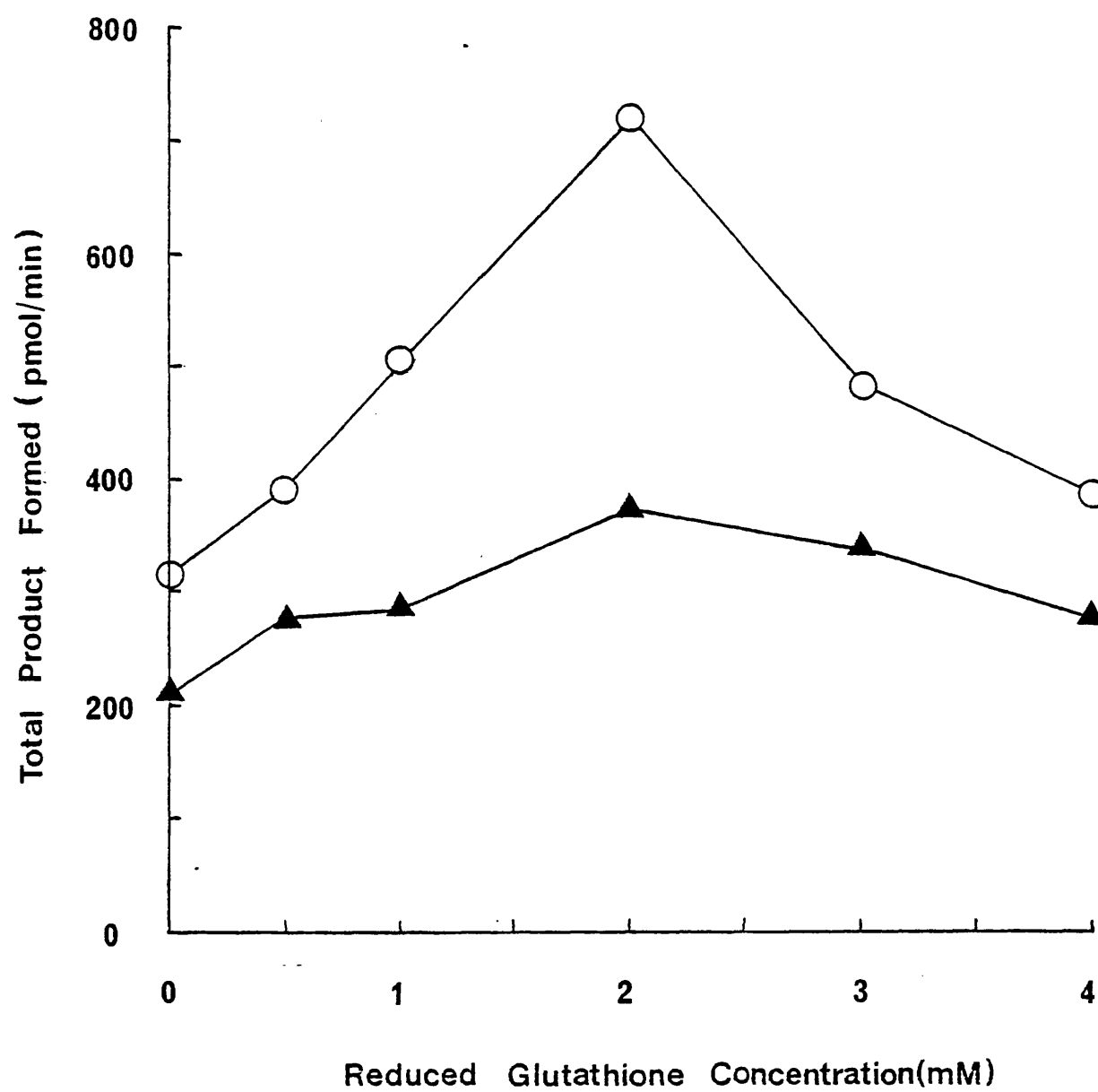


Figure 20. The effect of increasing concentrations of L-cysteine on the ability of decidual (O-O) and myometrial (▲-▲) microsomal preparations to synthesize PG-like products from AA. The optimal concentration for both decidual and myometrial microsomes was found to be 1mM.

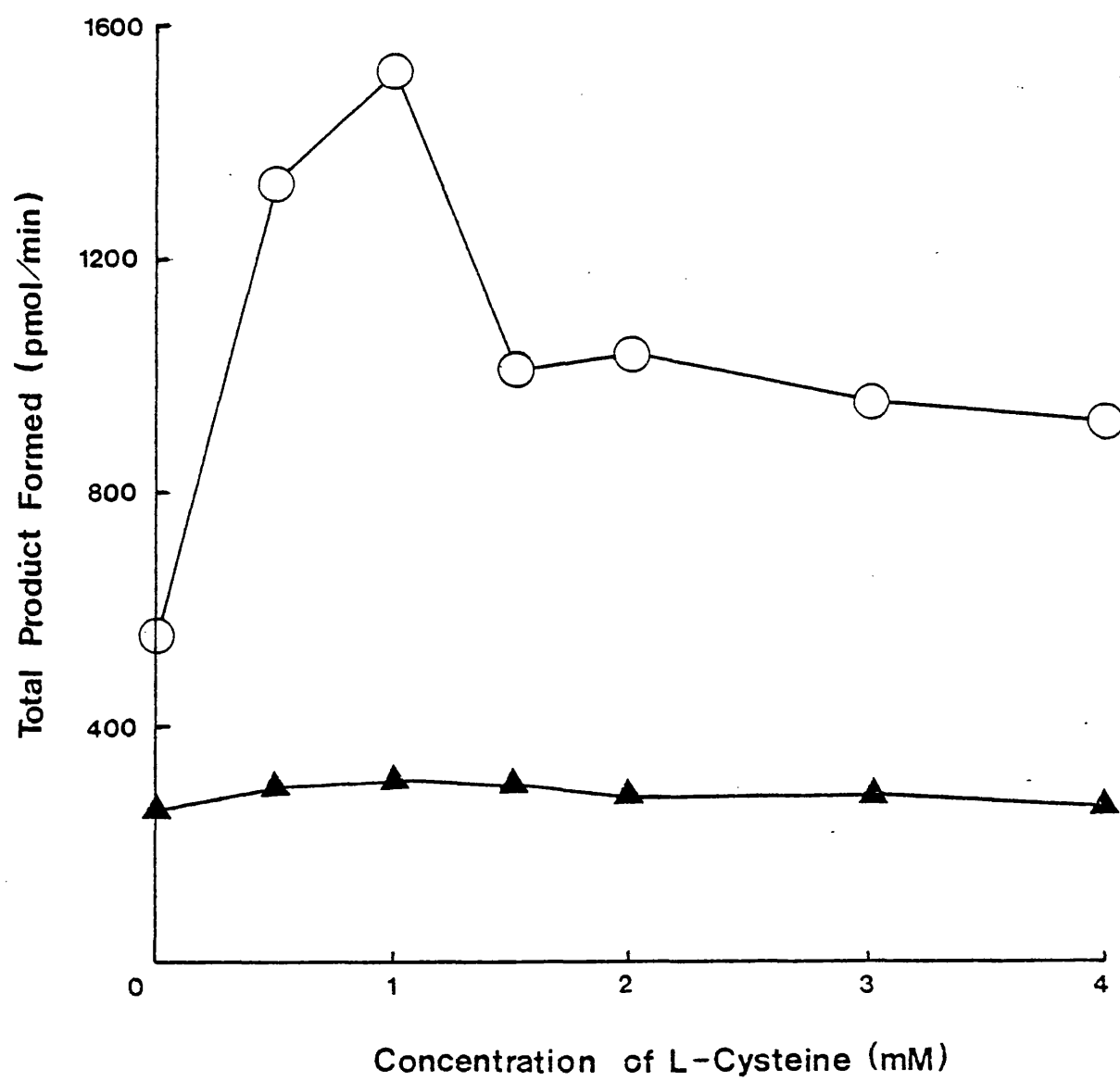
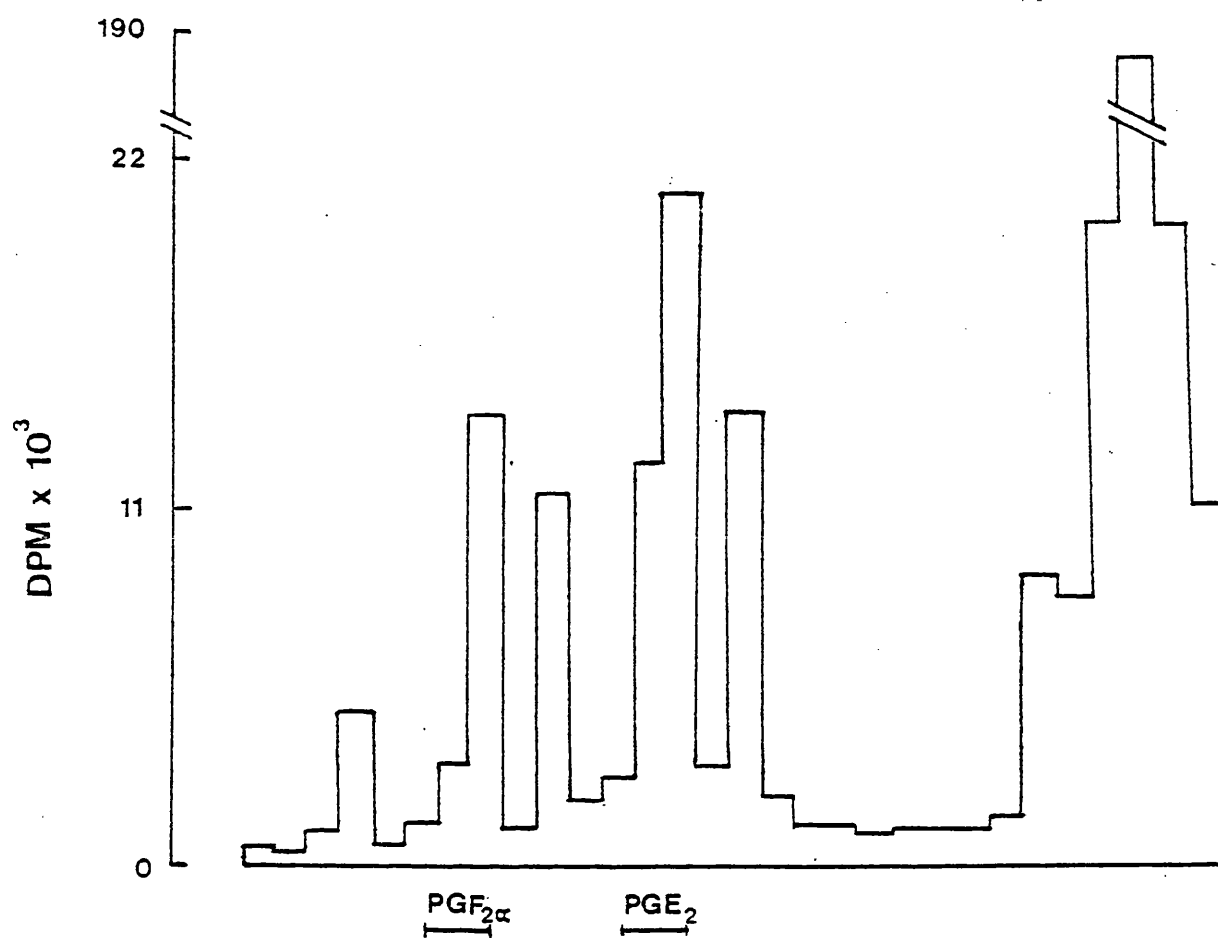


Figure 21. The influence of GSH and L-cysteine on AA metabolism by decidual microsomes. After the incubation the products were extracted and run using solvent system 1 (chloroform/methanol/acetic acid/water, 90:9:1:0.65). For the purpose of liquid scintillation counting the plate was divided into 0.5 cm zones. In the top chromatograph the decidual microsomes were incubated with GSH and L-cysteine and PG synthesis was normal. In the lower chromatograph GSH and L-cysteine were omitted from the incubation medium and product formation decreased.

GSH and L-Cysteine Present



GSH and L-Cysteine Absent

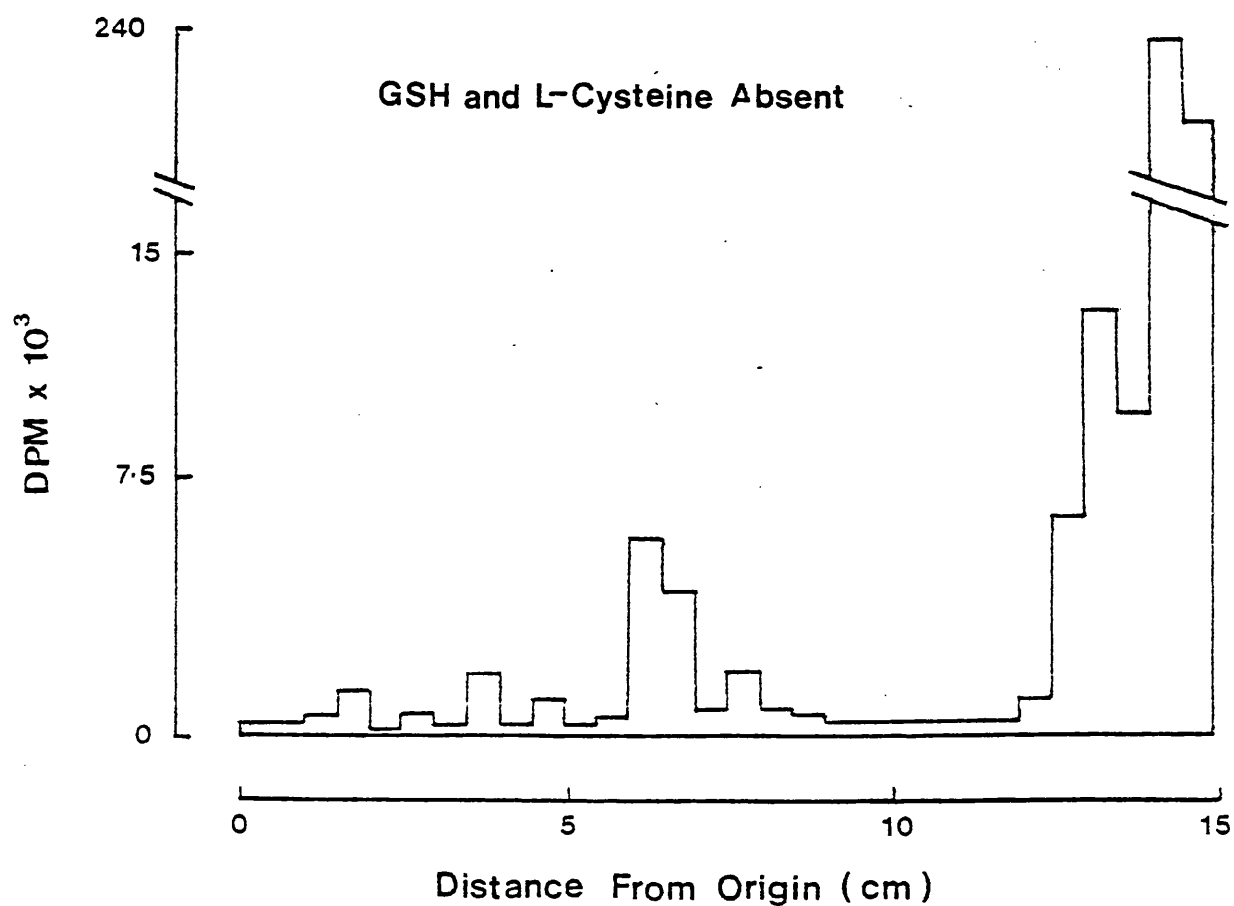
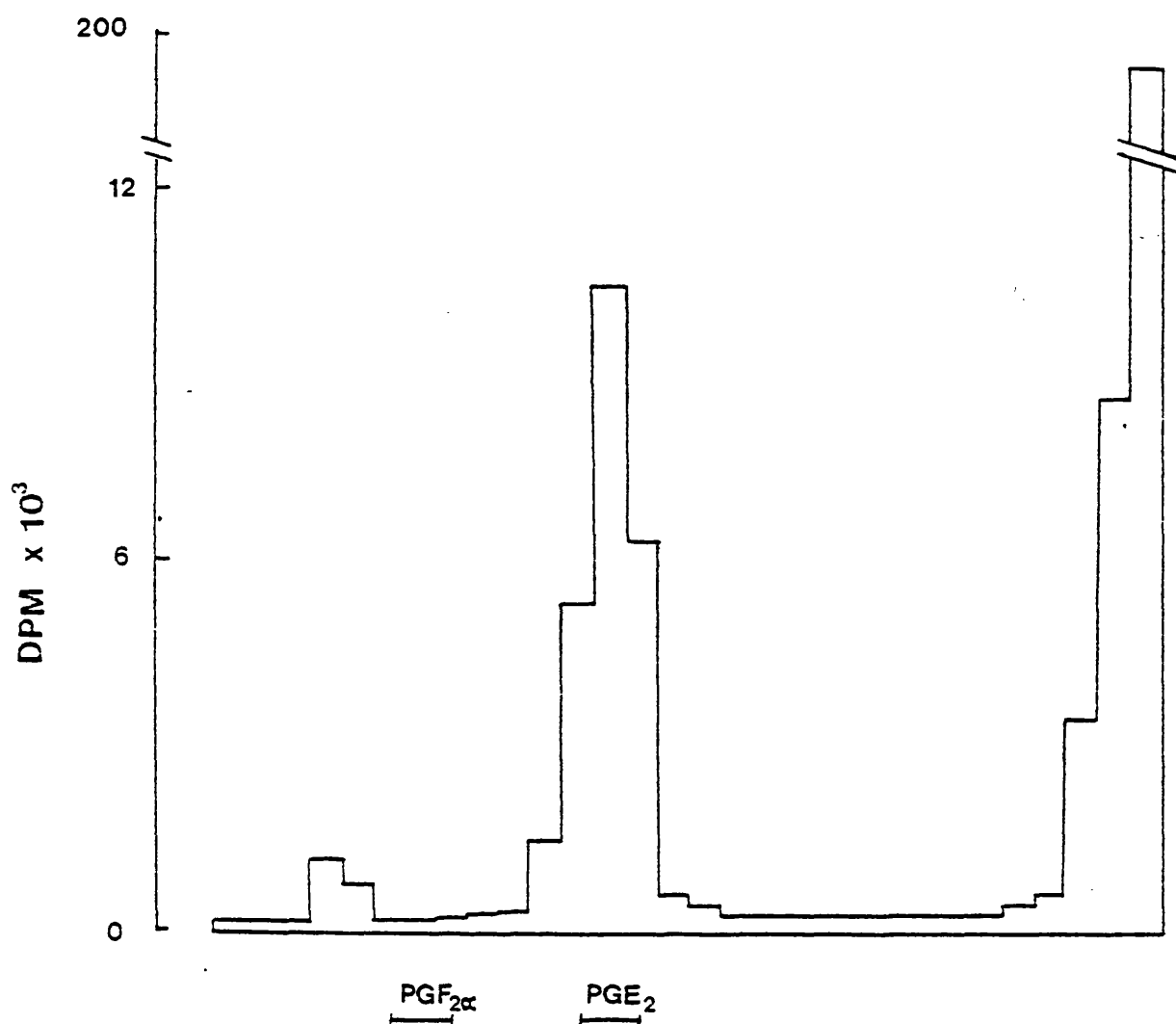
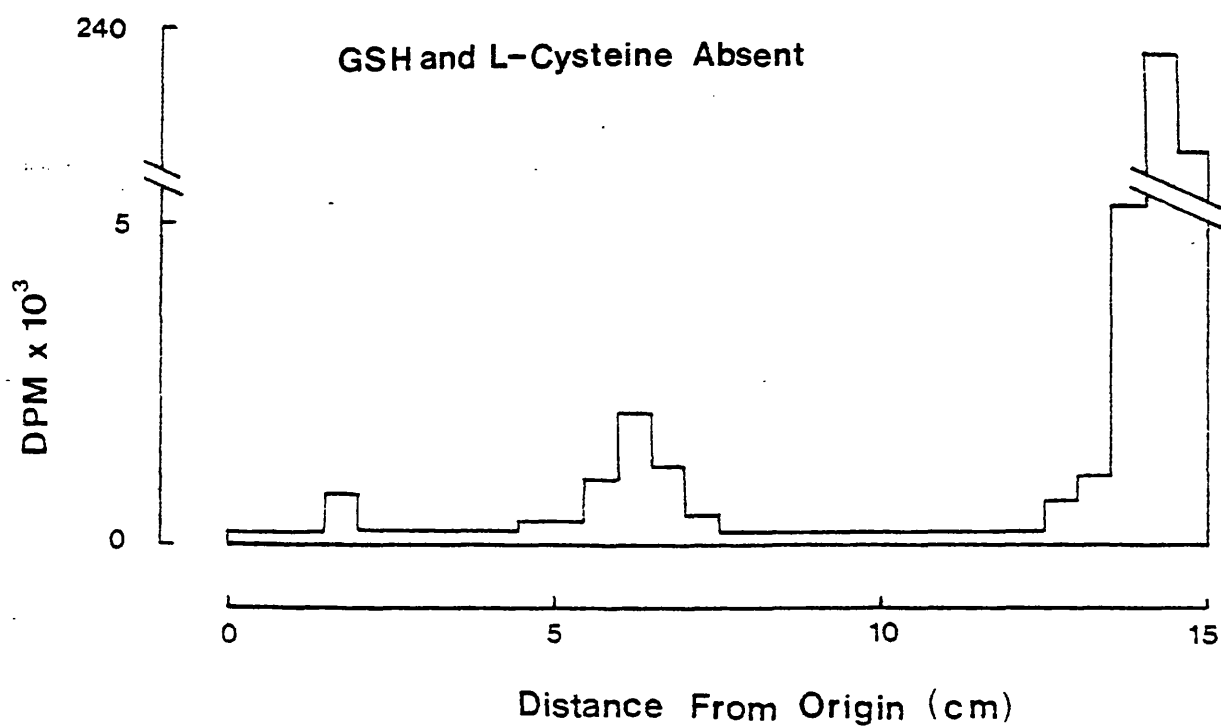


Figure 22. The influence of GSH and L-cysteine on AA conversion by myometrial microsomes. The extracts of the incubates were run in solvent system 1 (chloroform/methanol/acetic acid/water, 90:9:1:0.65). The plates were then divided into 0.5 cm zones for the purpose of counting the radioactivity present. In the upper chromatograph the myometrial microsomes were incubated in the presence of GSH and L-cysteine. In the lower chromatograph GSH and L-cysteine were omitted from the incubation mixture. It can be seen that the absence of the two cofactors drastically lowered PG synthesis.

GSH and L-Cysteine Present



GSH and L-Cysteine Absent



And absence of the relevant cofactors. The results can be seen in Table 3. This shows no difference in the proportions of the PG-like products produced by either decidual or myometrial microsomes when incubated with SH containing cofactors, compared to microsomes incubated in the absence of SH containing cofactors.

e) EDTA: It was found that for decidual microsomes that the addition of 1mM EDTA increased substrate conversion by 50% compared to incubates without EDTA. Above 1mM there was a slight decline in synthesis (7% reduction at 4mM). The effect of EDTA on myometrial microsomes was similar to that seen with decidual microsomes, but produced a 40% increase in synthesis when incubated with EDTA, and a decrease of 12.5% at 4mM EDTA. For routine assays therefore, a 1mM concentration of EDTA was used for both tissues.

vi) Storage of Decidual and Myometrial Microsomes: Initial attempts to store suspensions of decidual and uterine microsomes involved freezing 1ml aliquots of the resuspended microsomes in buffer at -20°C . It can be seen from Fig. 24, that this was not satisfactory, approximately 65% of the activity for both suspension was lost when stored overnight.

An attempt to prepare an acetone-pentane powder by the method of Wallach and Daniels (1971) was also unsuccessful, the activity of the resulting preparation from decidual and myometrial homogenates being very low, approximately 1nmole per 30 minutes incubation.

GSH L-cysteine	D E C I D U A				M Y O M E T R I U M	
	Total Synthesis nmoles	% COMPOSITION OF PG-LIKE PRODUCTS			Total Synthesis nmoles	% COMPOSITION OF PG-LIKE PRODUCTS PGE ₂ 6-oxo-PGF _{1α}
		PGF _{2α}	TxB ₂	PGE ₂		
+	22 ± 1	19 ± 1	11 ± 2	39 ± 3	8 ± 0.3	16 ± 2 69 ± 1
+	16 ± 3	17 ± 1	12 ± 2	35 ± 3	6 ± 2	13 ± 1 77 ± 2
-	13 ± 3	20 ± 2	9 ± 2	37 ± 3	7 ± 1	9 ± 1 78 ± 2
-	8 ± 0.4	15 ± 2	10 ± 2	40 ± 1	3 ± 0.4	14 ± 2 70 ± 2

Table 3. The effect of SH containing cofactors on proportions of products produced by incubates of decidual and myometrial microsomal suspensions. (Expressed as a % of the total PG-like products synthesized).

Figure 23. The effect of increasing EDTA concentrations on the synthesis of PG-like products by decidual (O-O) and myometrial (▲-▲) microsomes from AA. The optima for both microsomal fractions was 1mM, above this there was a slight decrease in PGs synthesized by both microsomal preparation. Below the optimal concentration of EDTA there was a sharp decrease in PG synthesis by both microsomal preparations.

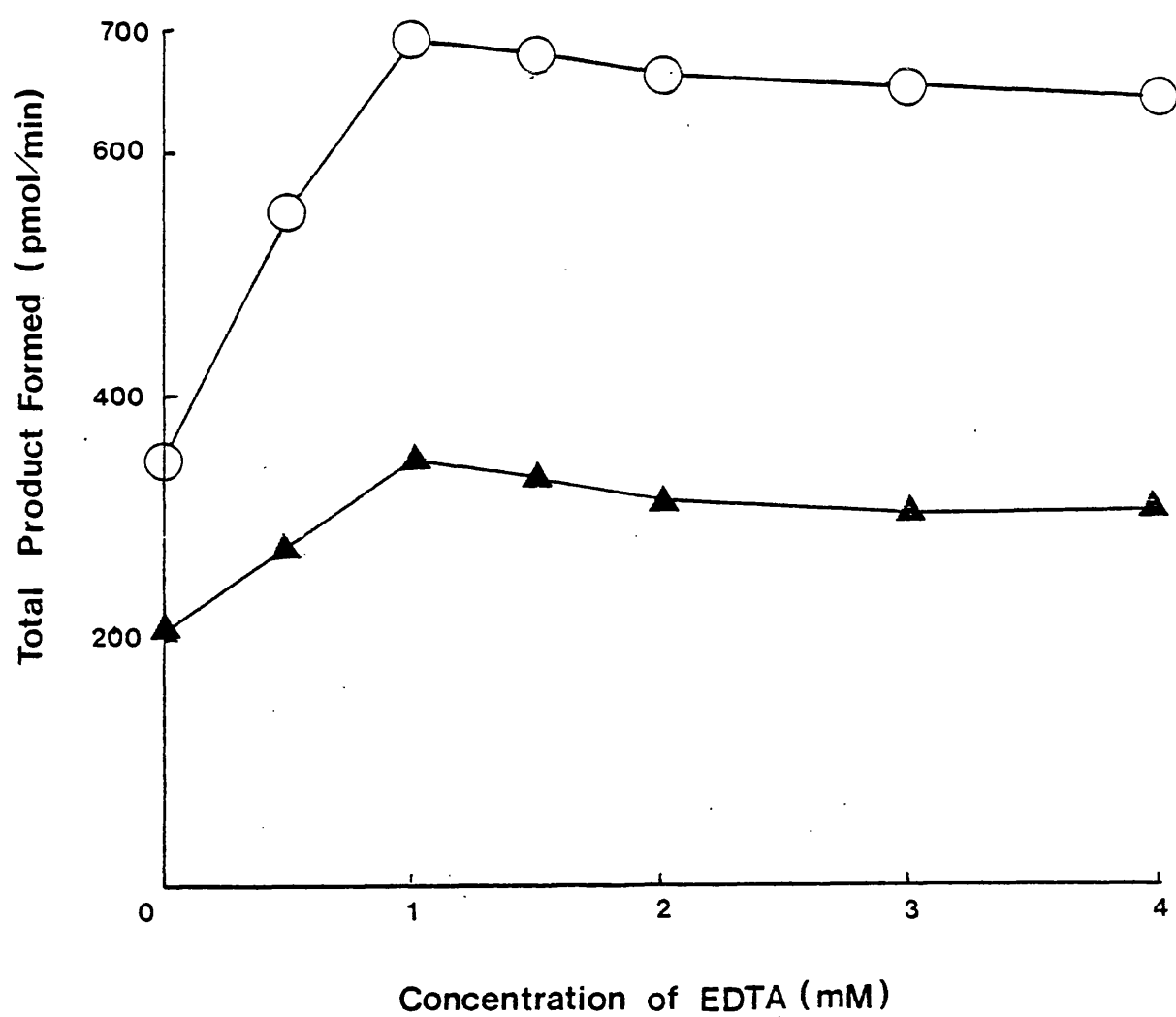
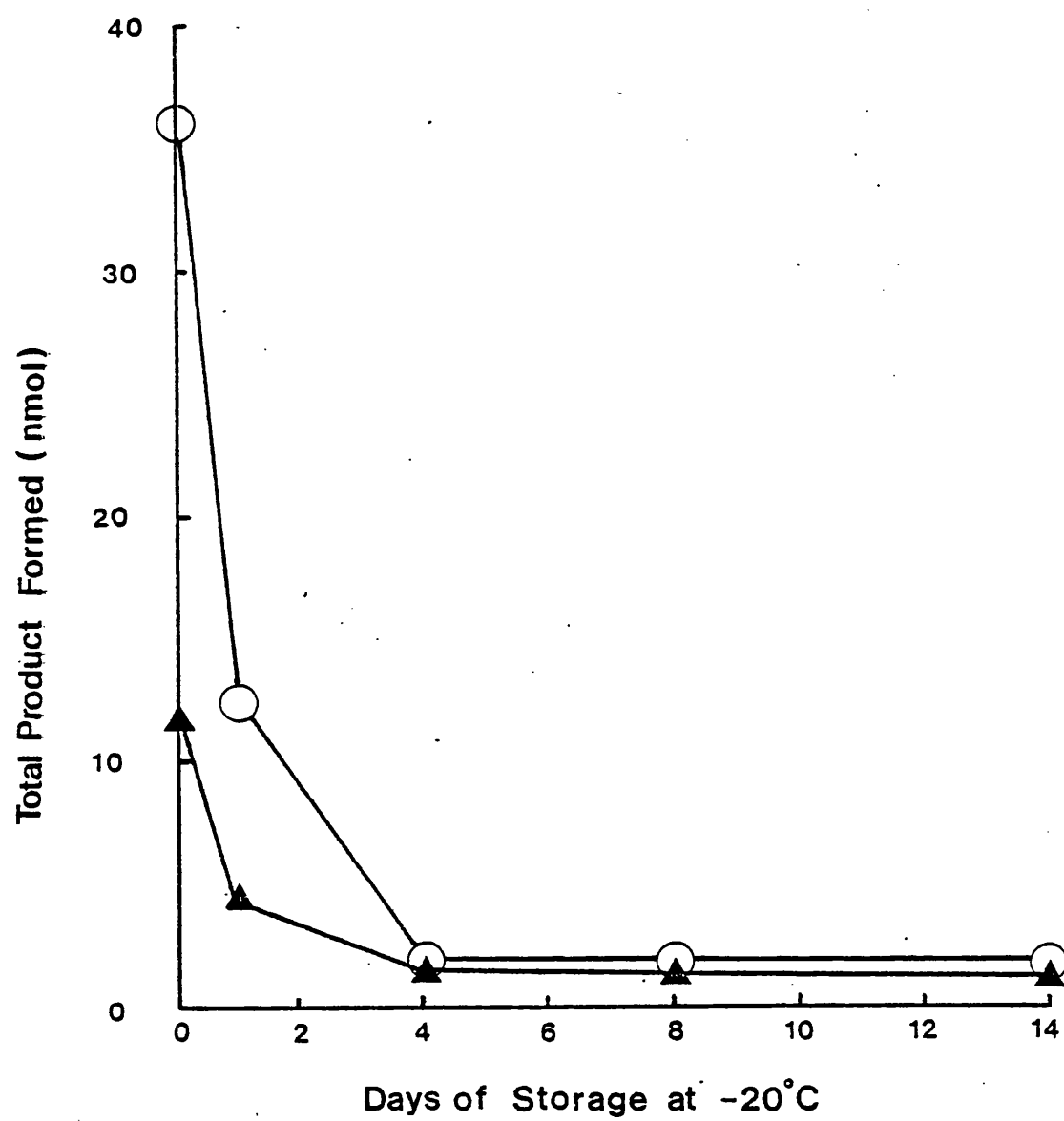


Figure 24. The effect of storage of decidual (O-O) and myometrial (▲-▲) microsomes at -20°C , in 1ml aliquots and the subsequent effects on PG-like products synthesized from AA when thawed at various time intervals. The storage of microsomal fraction of decidual and myometrial tissue in this manner caused a 65% loss of activity overnight.



3.5 Replacement of Hydroquinone by Other Cofactors

It was decided to investigate the possibility of replacing hydroquinone by other cofactors, e.g. the biogenic amines, adrenaline, noradrenaline, 5-hydroxytryptamine, and histamine, also L-ascorbic acid and a combination of L-tryptophan and haemoglobin. In several experiments with decidual and myometrial microsomes those peaks in the initial TLC with co-chromatographing components were scraped off, eluted, and rerun on the appropriate TLC system. The subsequent results reflected any change in the proportions of products synthesized.

Comparison of overall synthesis showed that for the decidual microsomes (Fig. 25) only 5-hydroxytryptamine could substitute equally for hydroquinone, with adrenaline and noradrenaline having about 50% of the stimulatory ability of hydroquinone. Histamine and the combination of L-tryptophan and haemoglobin proved particularly poor. L-ascorbic acid was better having approximately a quarter of the stimulatory activity of hydroquinone. In the case of myometrial microsomes (Fig. 26) using adrenaline, noradrenaline and 5-hydroxytryptamine in place of hydroquinone produced a 33-93% increase in AA metabolism (but this was only significant for noradrenaline). Histamine and the L-tryptophan-haemoglobin combination were again poor as cofactors. L-ascorbic acid stimulated the myometrial microsomes surprisingly well, and was almost comparable to the stimulation obtained with hydroquinone.

With decidual microsomes an increase in the proportion of $\text{PGF}_{2\alpha}$ was seen with all cofactors compared to hydroquinone, Table 4. With 5-hydroxytryptamine and histamine there was a slight decrease in TXB_2

Figure 25. Ability of different cofactors to substitute for hydroquinone as cofactor for AA metabolism in decidua microsomes. p Values are all relative to hydroquinone. * = $p < 0.05$, ** = $p < 0.02$, *** = $p < 0.0001$.

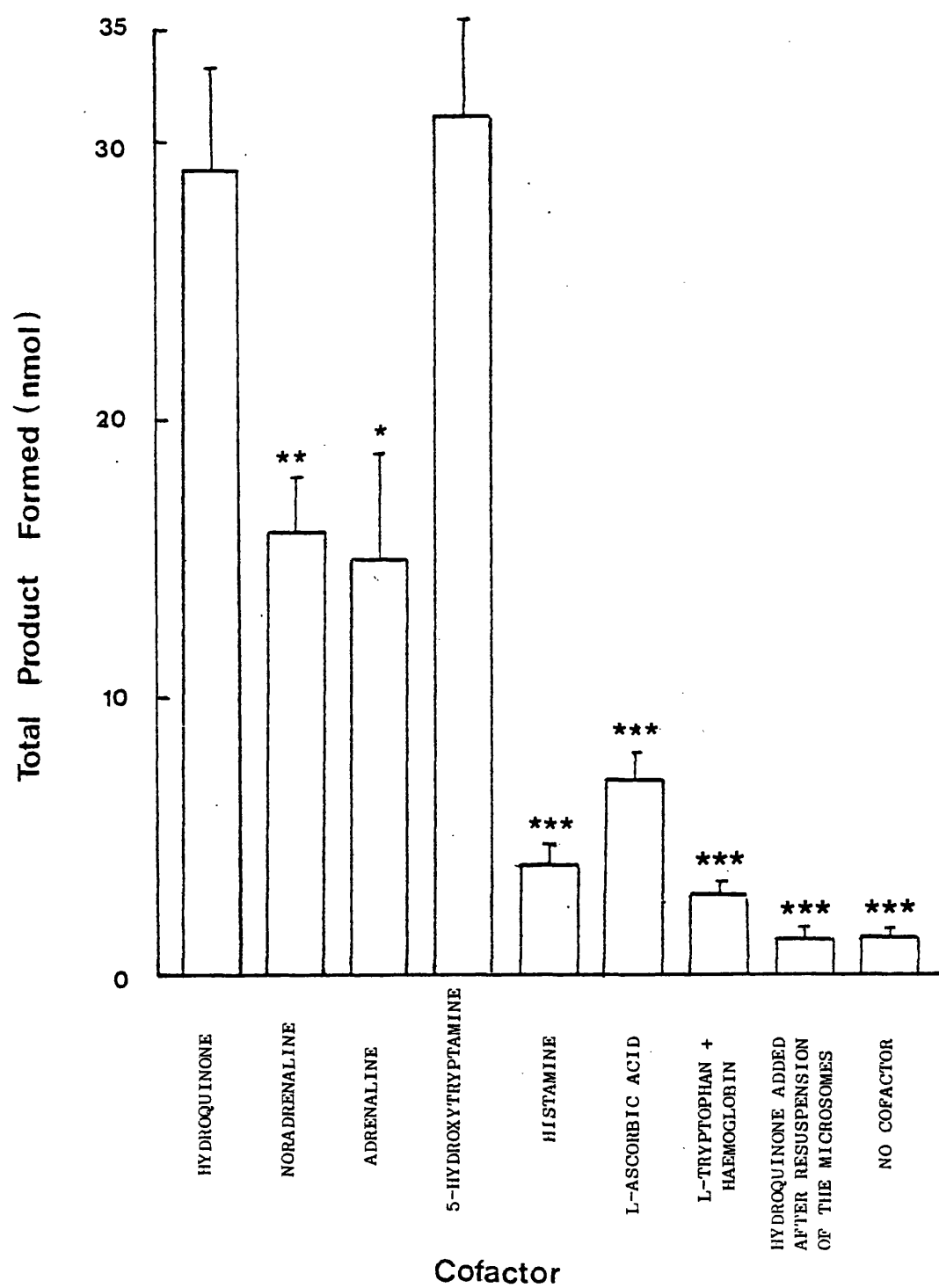


Figure 26. Ability of different cofactors to substitute for hydroquinone as a cofactor for AA metabolism in myometrial microsomes. p values are all relative to hydroquinone, * = $p < 0.05$, ** = $p < 0.02$, *** = $p < 0.001$.

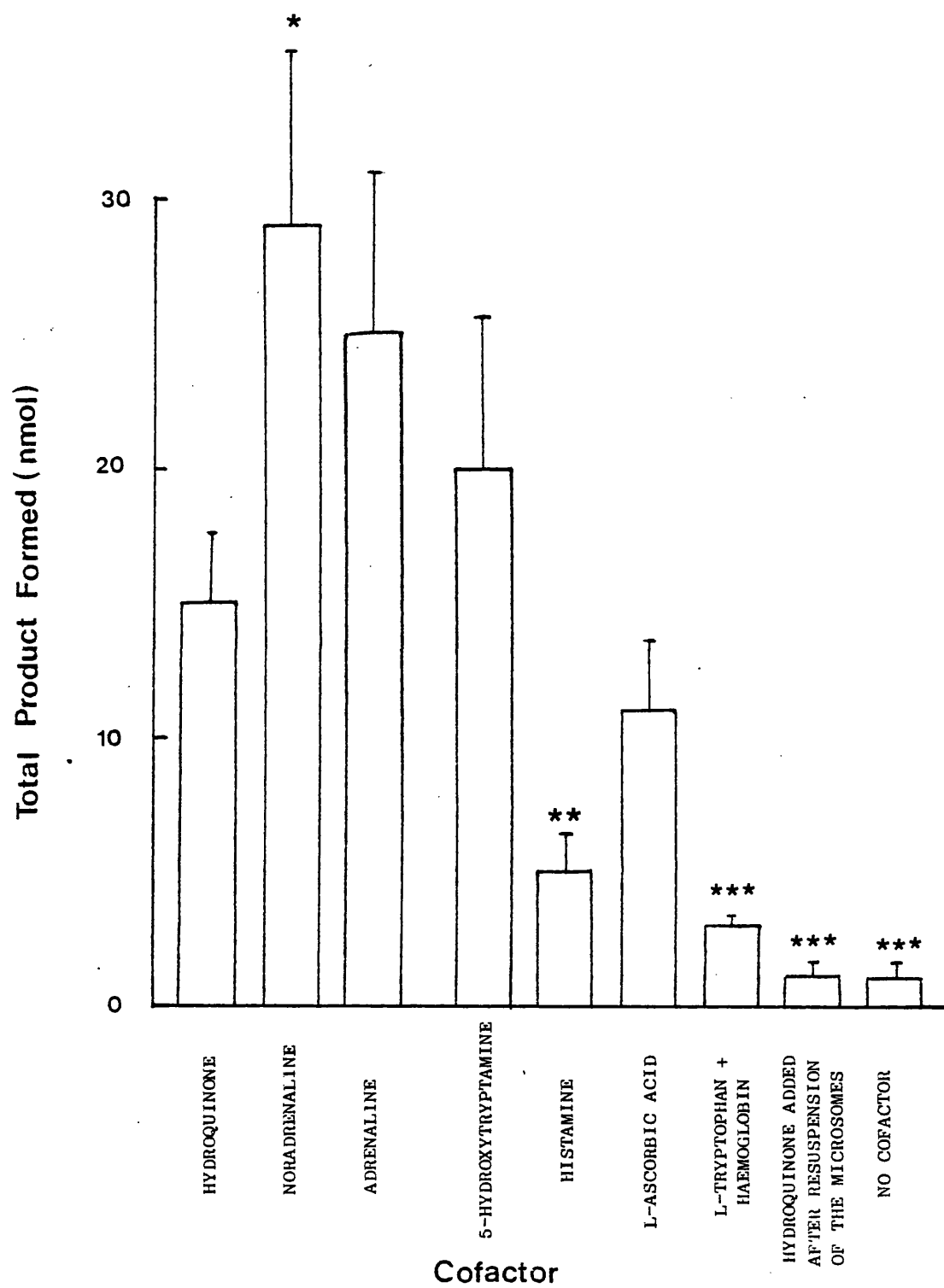


Table 4. The Effect of Different Cofactors used to replace Hydroquinone in Proportions of Products synthesized. (Expressed as a % of the total PG-like Products)

* = $p < 0.05$, † = $p < 0.01$, ‡ = $p < 0.001$.

COFACTOR	D E C I D U A					M Y O M E T R I U M	
	% COMPOSITION OF PG-LIKE PRODUCTS					% COMPOSITION OF PG-LIKE PRODUCTS	
	PGF _{2α}	TXB ₂	PGE ₂	6-oxo-PGF _{1α}	PGD ₂	PGE ₂	6-oxo-PGF _{1α}
Hydroquinone	15±1	12±1	36±4	15±2	10±2	8±0.5	83±1
Noradrenaline	26±2†	73±3	31±3	16±2	9±1	8±0.5	81±2
Adrenaline	26±2†	13±3	30±3	18±2	8±1	8±1	83±2
5-Hydroxytryptamine	27±4‡	7±0.4‡	32±3	17±2	8±1	12±2	74±2†
Histamine	21±4*	8±0.5†	32±3	22±5	10±3	12±2	74±3*
L-Ascorbic acid	21±2*	11±3	27±3	22±5	8±1	8±1	81±2
L-Tryptophan plus Haemoglobin	28±3‡	10±2	31±2	22±2	8±1	12±3	83±3

Table 5. The influence of cofactors on the proportions of PG-like products produced by decidual and myometrial microsomes (nmol/30 minutes).

Cofactor	D E C I D U A					M Y O M E T R I U M	
	COMPOSITION OF PG-LIKE PRODUCTS (nmol/30 minutes)					COMPOSITION OF PG-LIKE PRODUCTS (nmol/30 minutes)	
	PGF _{2α}	TXB ₂	PGE ₂	6-oxo-PGF _{1α}	PGD ₂	PGE ₂	6-oxo-PGF _{1α}
Hydroquinone	4.4	3.5	10.4	4.4	2.9	1.2	12.5
Noradrenaline	4.2	2.1	5.0	2.6	1.4	2.3	23.5
Adrenaline	3.9	2.0	4.5	2.7	1.2	2.0	20.3
5-hydroxytryptamine	8.4	2.2	9.9	5.3	2.5	2.4	14.8
Histamine	0.8	0.3	1.3	0.9	0.4	0.6	3.7
L-Ascorbic Acid	1.5	0.8	1.9	1.5	0.6	0.9	0.9
L-Tryptophan Plus Haemoglobin	0.8	0.3	0.9	0.6	0.2	0.4	2.5

formation as well. With myometrial microsomes the only significant change was seen with 5-hydroxytryptamine and histamine where there was a slight decrease in the percentage of 6-oxo-PGF_{1 α} formed, but with no significant increase in PGE₂ formation (Table 4). It must be noted that this may be due to losses incurred in the process of rechromatographing the product peaks, although this was consistent and low, in the order of $8.2 \pm 1.2\%$, $n = 39$, for the decidua, and $11.7 \pm 0.8\%$, $n = 39$ for the myometrium. Table 5, shows the contribution which each AA metabolite made to the total PG-like products formed, in nmole, by decidual and myometrial microsomes.

3.6 Inhibition of Uterine PG Synthetase by Non-Steroidal Anti-Inflammatory Drugs.

The results shown in Figs. 27 and 28 display the susceptibility of decidual and myometrial microsomal PG synthetase to a variety of non-steroidal anti-inflammatory drugs (NSAIDs). All NSAIDs were found to inhibit PG biosynthesis in a dose related manner for both preparations. The inhibition by NSAIDs was calculated from reduction in total product synthesized.

From the dose related inhibition of PG synthesis the dose of the drug which caused 50% inhibition (I.D.₅₀) was calculated graphically (see Table 6). This revealed that the most potent drug used was flurbiprofen, while the least potent was metopirone. From the I.D.₅₀s the relative potencies for the drugs were expressed on a molar basis relative to TYA for both decidual and myometrial preparations. The rank order of potency was found to be, in order of decreasing potency; flurbiprofen > meclofenamate > indomethacin > fenclozic acid > aspirin > alclofenac, for both decidual and myometrial PG synthetase.

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Figure 27. Inhibition of decidual PG synthetase in vitro by NSAIDs.

Each drug was preincubated for 5 minutes with the microsomal suspension, then incubated normally for 30 minutes under routine conditions. Drugs used were; flurbiprofen (◆-◆), meclofenamate (□-□), indomethacin (Δ-Δ), TYA (●-●), tranylcypromine (▲-▲), aspirin (■-■), alclofenac (◇-◇), and metopirone (O-O).

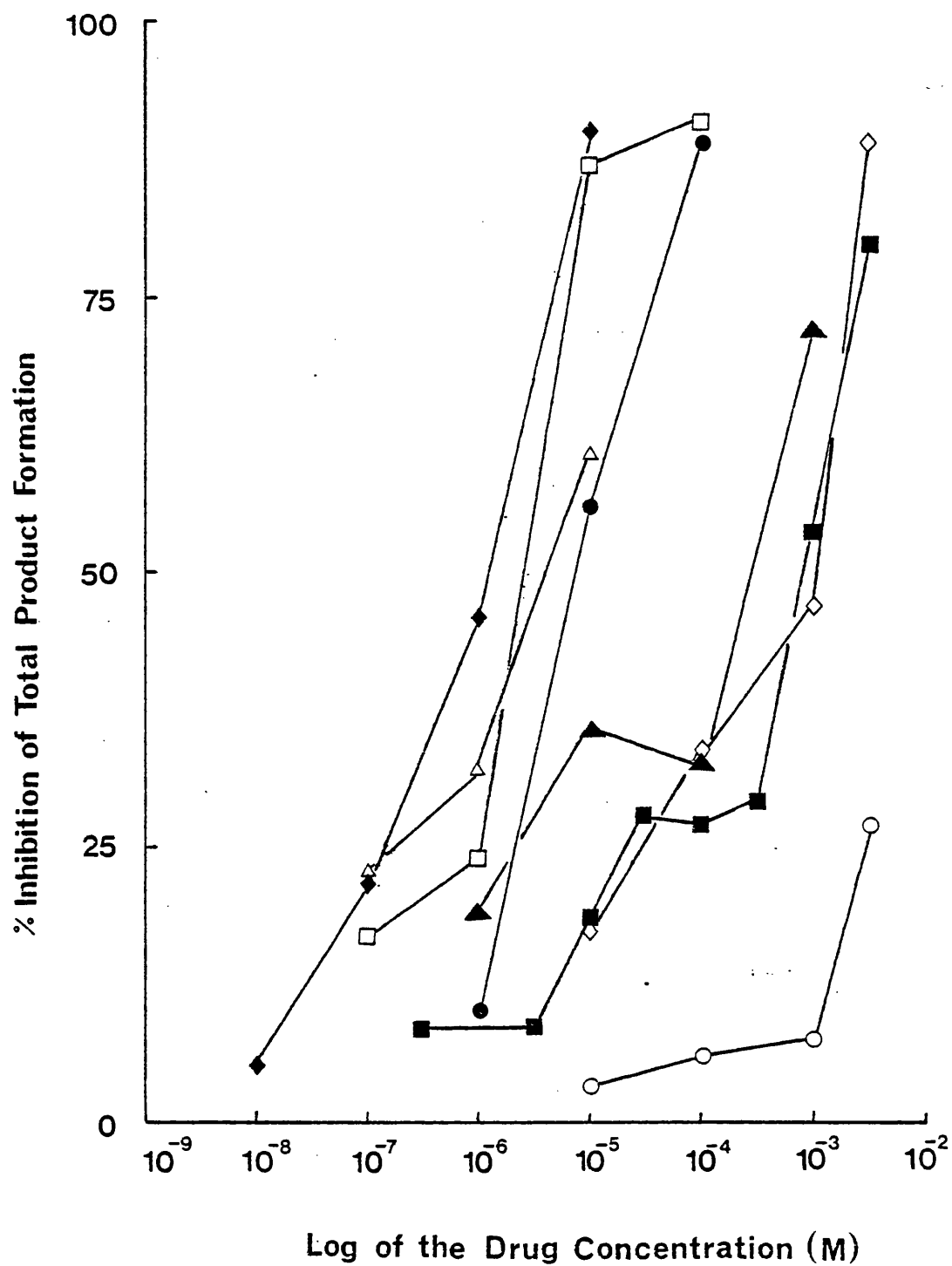


Figure 28. Inhibition of myometrial microsomal PG synthetase by NSAIDs. Each drug was preincubated for 5 minutes with the microsomal suspension and then incubated under routine conditions for 30 minutes. Drugs used were: flurbiprofen (◆-◆), meclofenamate (□-□), indomethacin (Δ-Δ), TYA (●-●), tranyclypromine (▲-▲), aspirin (■-■), alclofenac (◇-◇), and metopirone (O-O).

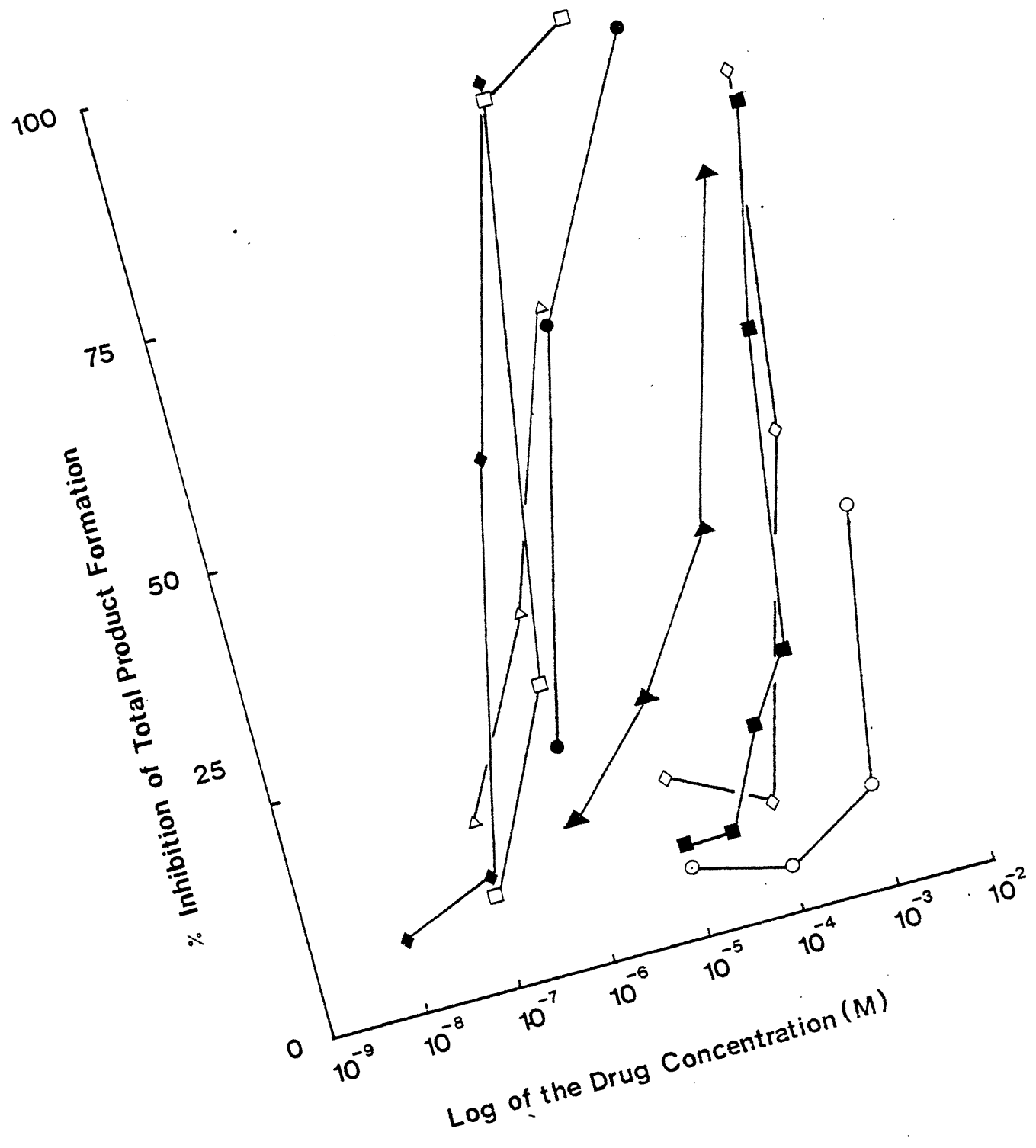


Table 6. I.D.₅₀ of NSAIDs incubated with uterine microsomes.

Drug	I. D. ₅₀	
	Decidua (M)	Myometrium (M)
Flurbiprofen	1.42×10^{-6}	8.29×10^{-7}
Meclofenamate	2.94×10^{-6}	2.86×10^{-6}
Indomethacin	4.56×10^{-6}	4.43×10^{-6}
TYA	1.05×10^{-5}	6.15×10^{-6}
Tranylcypromine	1.62×10^{-4}	2.73×10^{-4}
Fenclozic Acid	8.41×10^{-4}	5.62×10^{-4}
Aspirin	2.13×10^{-3}	7.89×10^{-4}
Alclofenac	2.58×10^{-3}	2.16×10^{-3}
Metopirone	3×10^{-3} , 26.9% inhibition	3×10^{-3} , 39.6% inhibition

Table 7. Relative Molar potency of NSAIDs incubated with Uterine microsomes. TYA, is taken as 1.00.

Drug	Relative Molar Potency for 50% inhibition of PG synthetase, relative to TYA	
	Decidua	Myometrium
Flurbiprofen	7.39	7.42
Meclofenamate	3.57	2.15
Indomethacin	2.30	1.39
TYA	1.00	1.00
Tranylcypromine	0.06	0.02
Fenclozic Acid	0.01	0.01
Aspirin	<0.01	0.01
Alclofenac	<0.01	<0.01

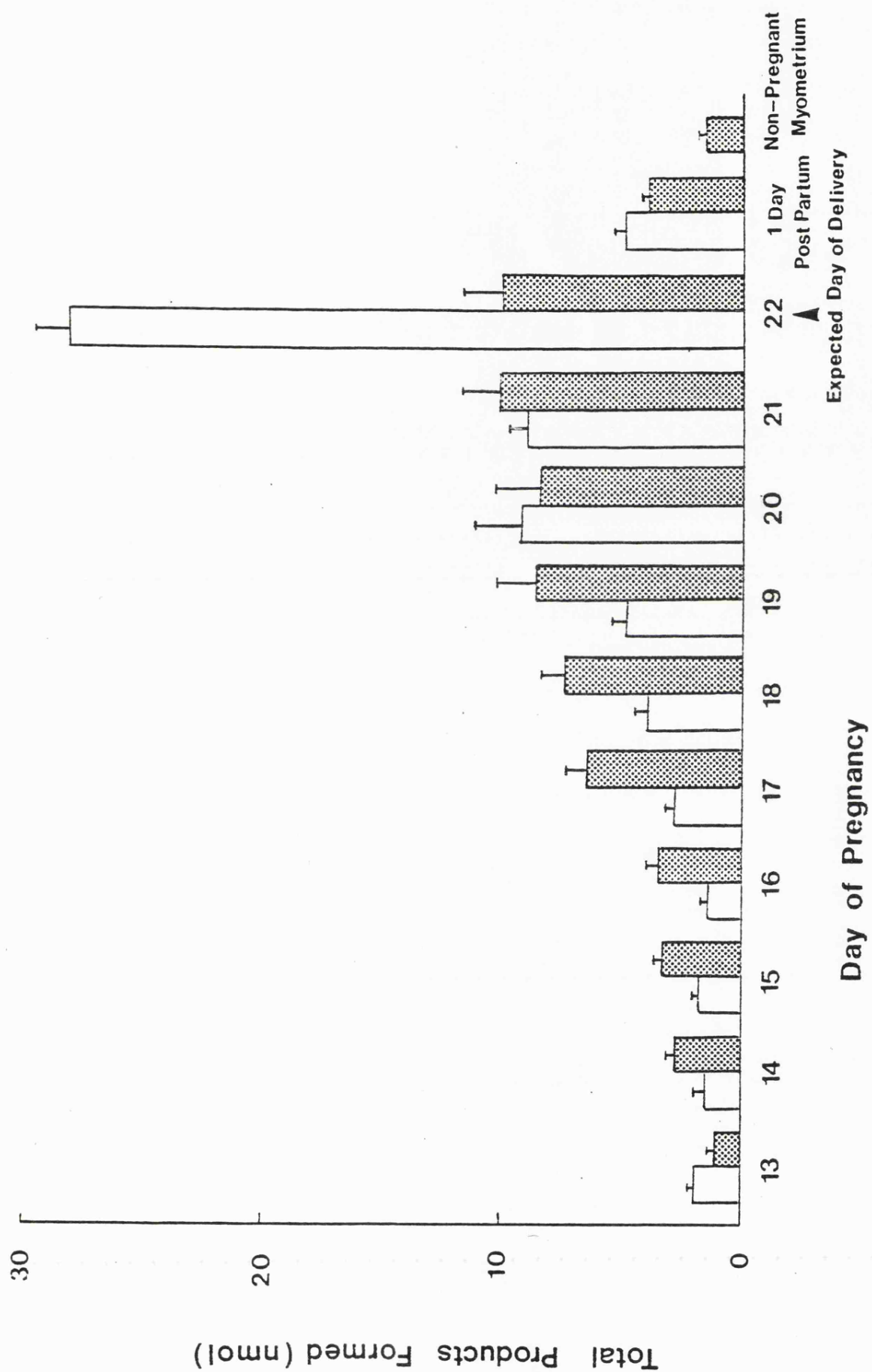
It was noticable from the dose response curves that the NSAIDs appear to be divided into two groups, flurbiprofen, meclofenamate, indomethacin and TYA in one group, and tranylcypromine, aspirin, fenclozic acid and alclofenac in the other. There is a break in the I.D.₅₀ values between tranylcypromine and TYA, 20 fold for the decidual and 50 fold for the myometrium.

Tranylcypromine was examined to see if there was any preferential inhibition of the AA metabolites synthesized by the uterine microsomes, especially the myometrial microsomes, where there may have been the possibility of a preferential inhibition of PGI₂ and hence 6-oxo-PGF_{1α}. But, it was found that the PGE₂-6-oxo-PGF_{1α} ratio was unchanged at a concentration of 10⁻⁴ M tranylcypromine (which gave 42% inhibition), the proportion of each was 14.4 ± 3.2% PGE₂ and 76.5 ± 5.3% 6-oxo-PGF_{1α}. Metopirone primarily a steroid biosynthesis inhibitor was also tried as a PG synthetase inhibitor, having been reported to stimulate PGF_{2α} formation in crude homogenates of day 22 pregnant rat uteri (Parnham and Sneddon, 1975). However, with decidual and myometrial microsomes it was found that metopirone was a poor PG synthetase inhibitor, causing only 27% and 40% inhibition of PG synthesis by decidual and myometrial microsomes respectively with a dose of 3 x 10⁻³ M. The relative proportions of products also remained unchanged.

3.7 The Influence of the Day of Pregnancy on PG Biosynthesis

It was found that between days 13 to 19 pregnancy that myometrial microsomes produced considerably more PGs than decidual microsomes (Fig. 29). The quantity of PGs synthesized by decidual and myometrial microsomes was similar on days 20, 21 and 22 of pregnancy. The peak of myometrial PG synthetic activity was on days 19-22, and dropped

Figure 29. The influence of the day of pregnancy on total PG synthesis by decidual (□) and myometrial (▣) microsomes during a 30 minute incubation. It can be seen that maximum PG synthesis by decidual microsomes occurs on day 22 of pregnancy. While maximum PG synthesis for myometrial microsomes is spread over days 19-22. Synthetic ability for both decidual and myometrial microsomes decreased one day post partum.



sharply one day post partum. While the decidual microsomes synthetic ability peaked dramatically on day 22, increasing the quantity of PGs synthesized nearly threefold, which one day post partum was abolished returning to levels comparable to the synthetic capacity of myometrial microsomes.

In the case of the myometrial microsomes although there is an increase in the quantity of PG synthesized the relative proportions of each product remained practically unchanged. 6-oxo-PGF_{1α} was always found to be the major product constituting 76-85% of the total PGs synthesized. However, for decidual microsomes there was a considerable variation in the proportions of the different PGs synthesized (Table 8). Before day 21 of pregnancy 6-oxo-PGF_{1α} is the major product, not until day 21 does PGE₂ become the major product. The proportion of PGD₂ was fairly constant between 3-6% which rose to 9% on day 22 and decreased again one day post partum. The proportion of TXB₂ was fairly steady between days 13-19 of pregnancy being between 4-7%. This rose to between 11-13% on days 20-22, and also decreased post-partum (5.5%). The proportion of PGF_{2α} fluctuated throughout the days of pregnancy studied, being 32% on day 13, 11% on day 17, increasing again to 27% on day 21, falling to 18% on day 22. However, even with these fluctuations the quantity of PGF_{2α} synthesized on day 22 was 50% up on that synthesized by the decidua on day 21 (table 9). In general terms the synthesis of all products of AA metabolism by decidual microsomes increased as pregnancy progressed and decreased after parturition. Figure 30 shows the decidual C and D peaks and the myometrial product peak from solvent system 1 (chloroform/methanol/acetic acid/water, 90:9:1:0.65) rechromatographed in solvent system 3 (organic phase of ethyl acetate/iso-octane/acetic acid/water, 11:5:2:10). This demonstrates the

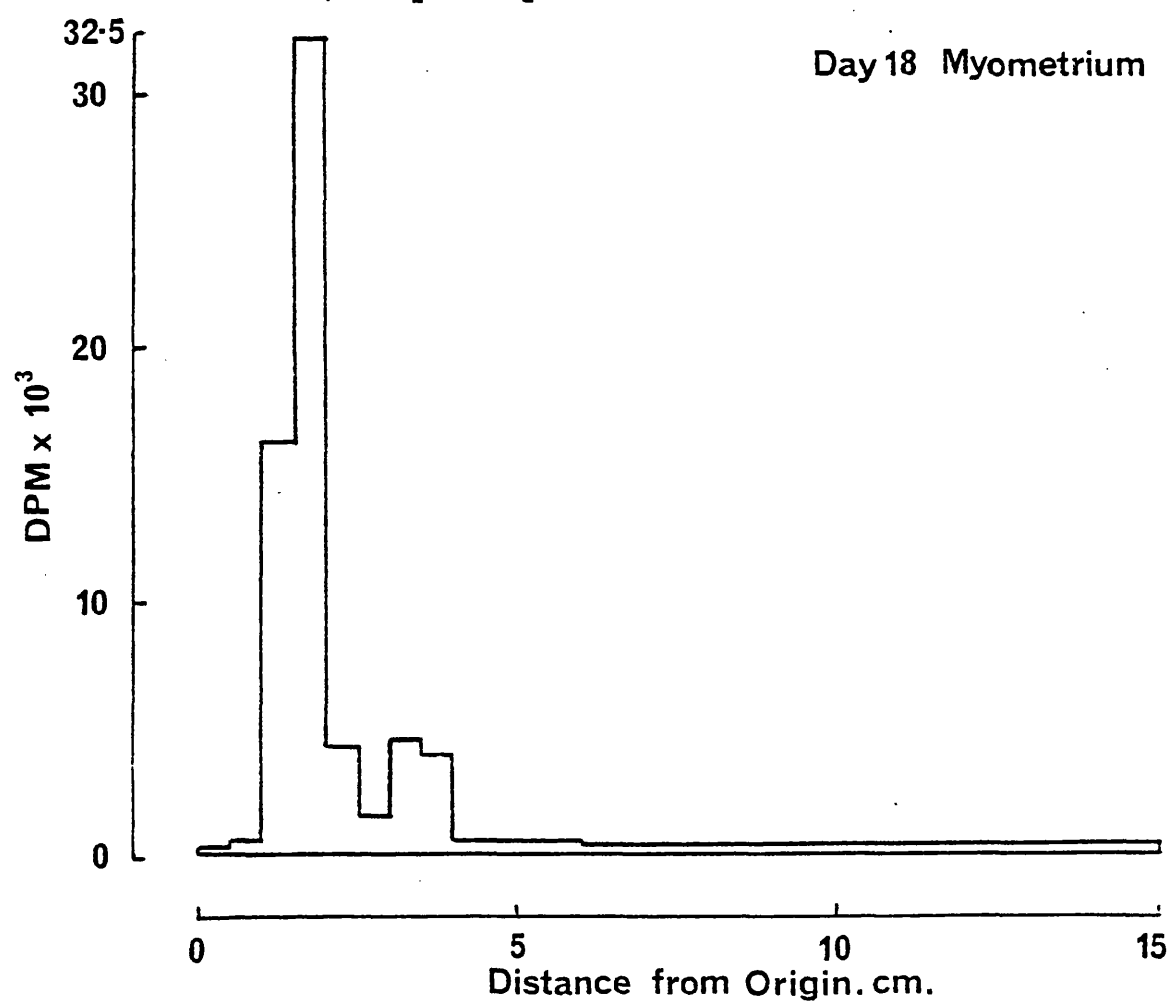
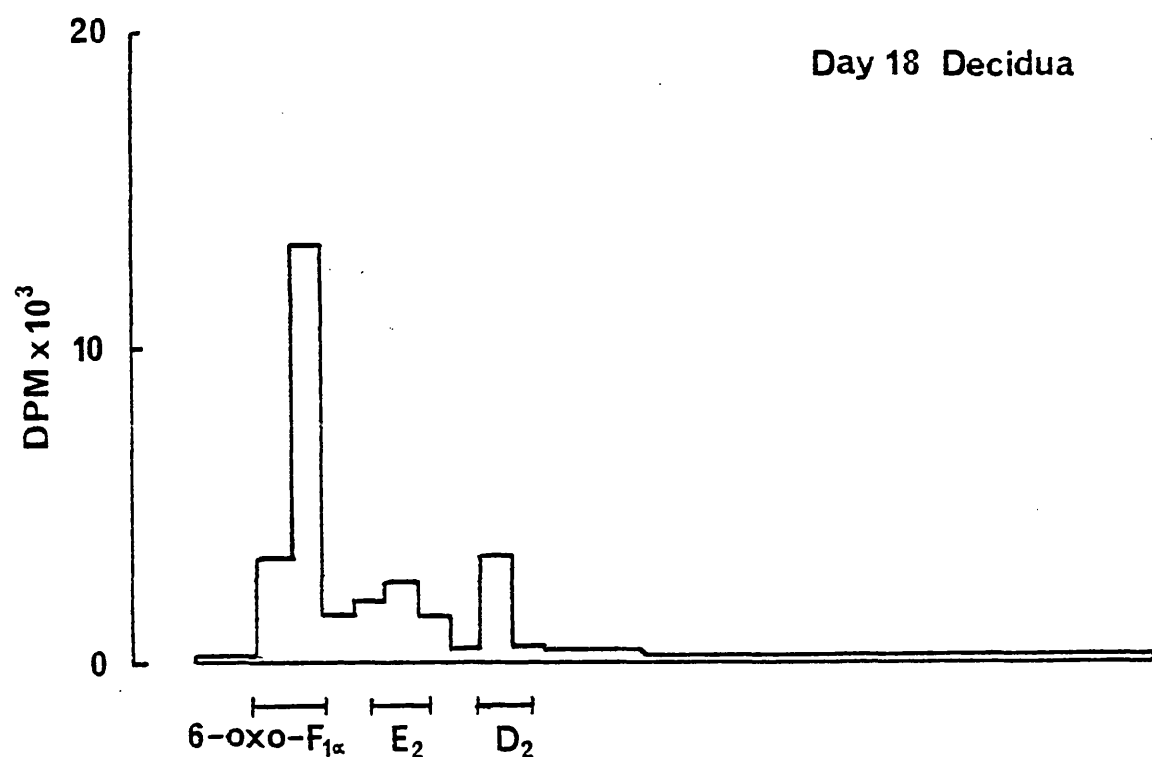
Table 8. Influence of the Day of Pregnancy on Proportions of PGs produced by uterine Microsomes. (Expressed as a % of total PG-like product).

Day of Pregnancy	D E C I D U A					M Y O M E T R I U M	
	% Composition of PG-like Products					% Composition of PG-like Products	
	PGF _{2α}	TXB ₂	PGE ₂	6-oxo-PGF _{1α}	PGD ₂	PGE ₂	6-oxo-PGF _{1α}
13	32.1±1.8	5.1±0.3	21.6±0.3	34.9±3.0	5.8±2.0	14.3±2.0	76.1±0.9
14	23.6±2.2	6.7±0.4	26.4±0.5	35.2±3.5	4.6±0.1	9.7±1.6	76.1±1.8
15	12.3±1.3	6.1±0.7	22.9±3.8	46.1±1.6	5.3±0.6	14.7±2.0	78.7±3.0
16	16.0±3.4	7.4±0.2	21.2±4.7	38.6±2.8	4.7±0.8	15.9±1.8	76.5±3.2
17	11.5±2.1	3.9±0.1	24.2±3.5	48.6±1.7	4.3±1.1	15.8±2.9	80.7±2.2
18	15.2±3.0	5.4±1.5	25.9±1.4	47.8±2.1	4.0±0.2	8.8±1.9	85.2±3.3
19	15.7±1.1	3.7±0.9	25.2±2.8	45.7±2.5	4.5±0.3	17.3±2.3	78.2±2.5
20	26.5±2.1	13.2±2.4	18.5±0.9	33.5±0.6	3.1±0.2	10.9±2.6	85.3±1.6
21	27.5±2.6	11.5±2.6	30.3±4.9	24.1±4.1	6.1±0.9	13.6±2.8	82.4±1.8
22	17.6±0.8	11.6±1.8	39.2±1.8	18.5±1.8	9.2±0.9	10.1±1.1	79.0±1.9
1 day post partum	13.8±2.5	5.5±1.3	37.5±2.3	24.0±2.0	6.5±1.8	13.1±2.9	82.4±2.5

Table 9. The influence of the day of pregnancy on actual quantity of each PG-like product synthesized by decidual and myometrial microsomes during a 30 minute incubation.

DAY OF PREGNANCY	D E C I D U A					M Y O M E T R I U M	
	COMPOSITION OF PG-LIKE PRODUCTS (nmole/30 minutes)					COMPOSITION OF PG-LIKE PRODUCTS (nmole/30 minutes)	
	PGF _{2α}	TXB ₂	PGE ₂	6-oxo-PGF _{1α}	PGD ₂	PGE ₂	6-oxo-PGF _{1α}
13	0.62	0.10	0.42	0.68	0.11	0.16	0.85
14	0.34	0.10	0.38	0.50	0.07	0.26	2.02
15	0.22	0.11	0.41	0.82	0.09	0.48	2.59
16	0.20	0.09	0.26	0.48	0.06	0.53	2.56
17	0.30	0.10	0.63	1.27	0.11	1.02	5.19
18	0.60	0.21	1.02	1.88	0.16	0.65	6.28
19	0.75	0.18	1.20	2.17	0.21	1.50	6.75
20	2.43	1.	1.70	3.07	0.28	0.92	7.17
21	2.42	1.01	2.66	2.12	0.54	1.37	8.32
22	4.96	3.27	11.05	5.22	2.59	1.03	8.06
1 day post partum	0.66	0.25	1.79	1.15	0.31	0.50	3.13

Figure 30. Thin layer chromatograph of decidual peaks C and D (upper chromatograph) and myometrial product peak (lower chromatograph) from a TLC run in solvent system 1 (chloroform/methanol/acetic acid/water, 90:9:1:0.65) rechromatographed in solvent system 3 (organic phase of ethyl acetate/iso-octane/acetic acid/water, 11:5:2:10) to demonstrate the difference in decidual PG production on day 18 of pregnancy.



difference in PG production by decidual microsomes on day 18 of pregnancy and compares it with myometrial PG synthesis on the same day.

3.8 Microsomal Preparation of Myometrium Lying Under the Placentae (MUP) and Placentae

Microsomal preparations of the MUP and the placentae themselves were investigated for their ability to synthesize PGs under routine conditions established for decidual and myometrial microsomes. This revealed that the MUP and the placentae had little ability to synthesize PGs from the exogenous substrate present in the incubation media (Fig. 31). Total PG synthesis by MUP was $1.63 \pm 0.18\%$ or 1.66 ± 0.19 nmoles, ($n = 24$), for the placentae $0.60 \pm 0.20\%$ or 0.61 ± 0.20 nmoles, ($n = 5$), over 30 minutes. Because of the low conversion of substrate to PGs, MUP sites were removed before preparation of decidual and myometrial microsomes to avoid dilution of the active PG synthesizing microsomes.

3.9 The Effect of Microsomal Supernatants on AA metabolism by Uterine Microsomes

It has long been known that there is a heat stable endogenous cofactor(s) present in the microsomal supernatant (see introduction). Thus an investigation was carried out to see if this endogenous cofactor(s) could replace hydroquinone in the incubation medium. The experiments were performed in hydroquinone-free buffer, with the respective supernatants being added back to the decidual and myometrial microsomes before resuspension. Half of the supernatant had been previously boiled for 2 minutes. Control incubations with the normal buffer were also carried out. The results shown in

Figure 31. The PG synthesizing capacity of microsomal preparations of various uterine tissues and placenta during a 30 minute incubation under standard conditions.

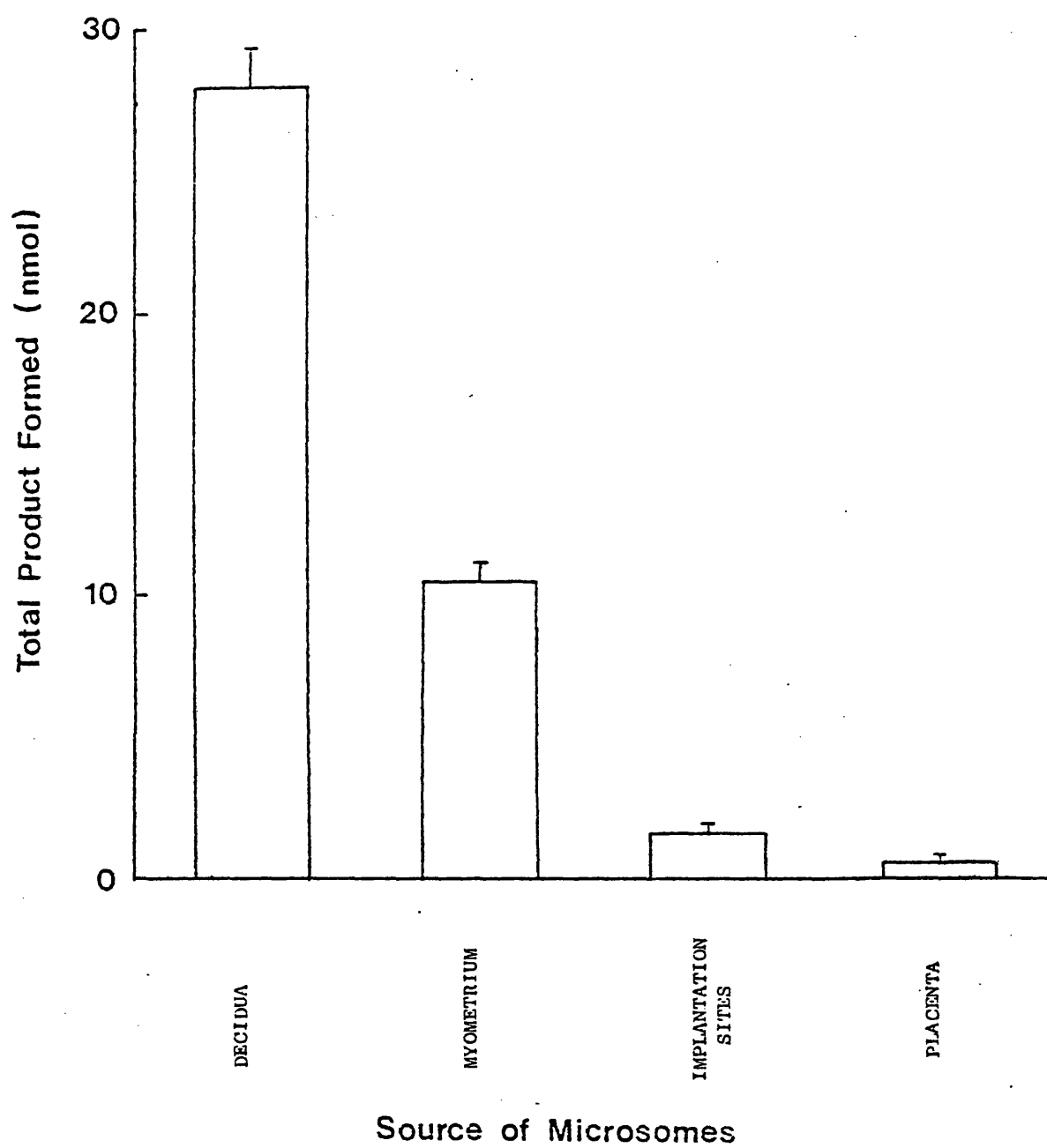


Table 10 Inhibitory activity of decidual and myometrial microsomal supernatants on PG synthesis by uterine microsomes

Source of Microsomes	Total Product Formed (nmol)		
	Control	Untreated Supernatant	Boiled Supernatant
Decidua	21.9±0.9	0.63±0.01	3.8±0.4
Myometrium	9.4±0.8	0.46±0.05	5.0±0.2

Table 10 indicate that the supernatants from the decidual and myometrial microsomes virtually abolished AA metabolism by their respective microsomes. Boiling of the supernatant samples before addition to the microsomes reversed this inhibition to some extent.

Further experiments were carried out under routine conditions i.e. the incubation mixtures containing hydroquinone and the microsomal supernatants were added in various volumes to the incubation mixture. The following combinations were tested, (1) addition of myometrial microsomal supernatant of myometrial microsomes (Fig. 32), (2) addition of decidual microsomal supernatant to decidual microsomes (Fig. 33), (3) addition of placental microsomal supernatant to both myometrial and decidual microsomes (Fig. 34). It was found with all three microsomal supernatants that there was a volume dependent inhibition of PG synthesis by decidual and myometrial microsomes. However, the degree of inhibition of PG synthesis caused by equivalent volumes of microsomal supernatants varied i.e. 100 µl of myometrial microsomal supernatant inhibited myometrial microsomes by 44%, while 100 µl of decidual microsomal supernatant inhibited decidual microsomes by only 23% and 100 µl of placental microsomal supernatant inhibited myometrial and decidual microsomes by 48% and 45% respectively. This inhibitory activity was abolished if the supernatants were boiled for two minutes.

Figure 32. Inhibition of myometrial PG production by myometrial
microsomal supernatant

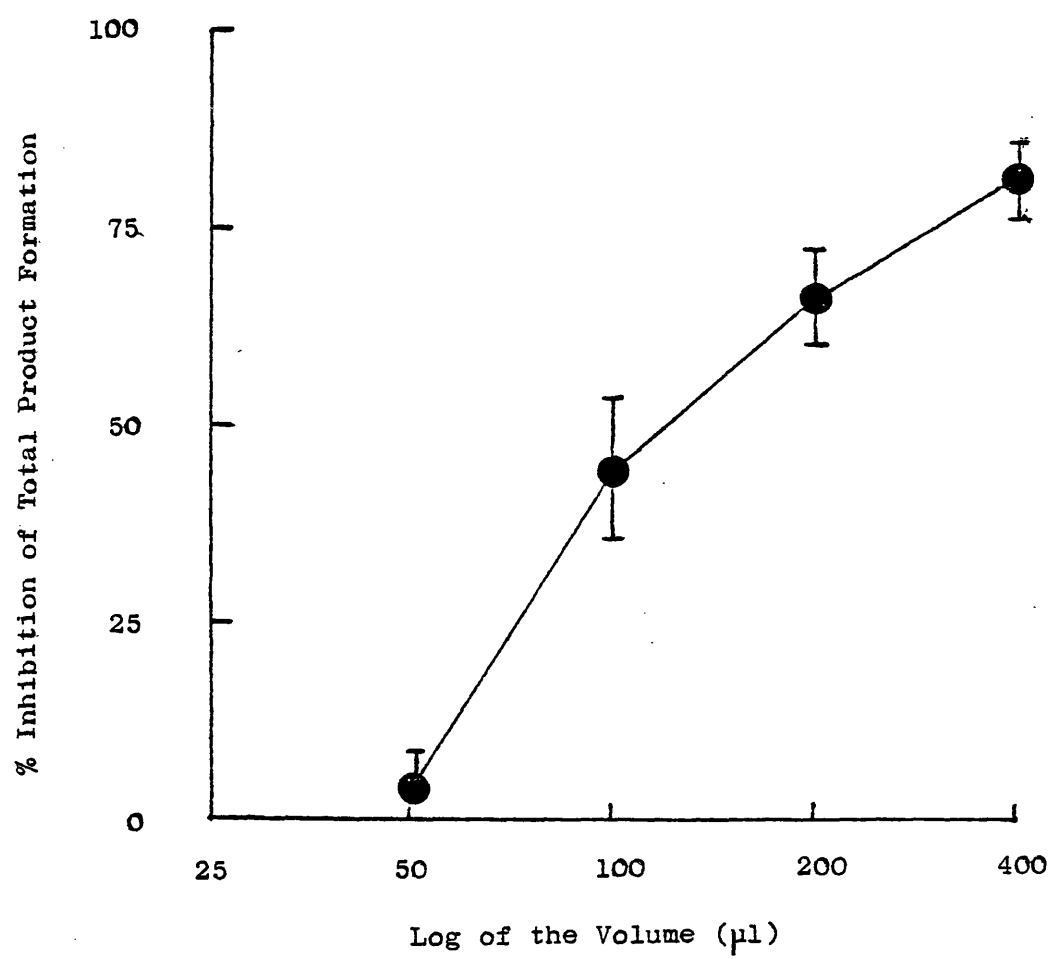


Figure 33. Inhibition of decidual microsomal PG synthesis by
decidual microsomal supernatant

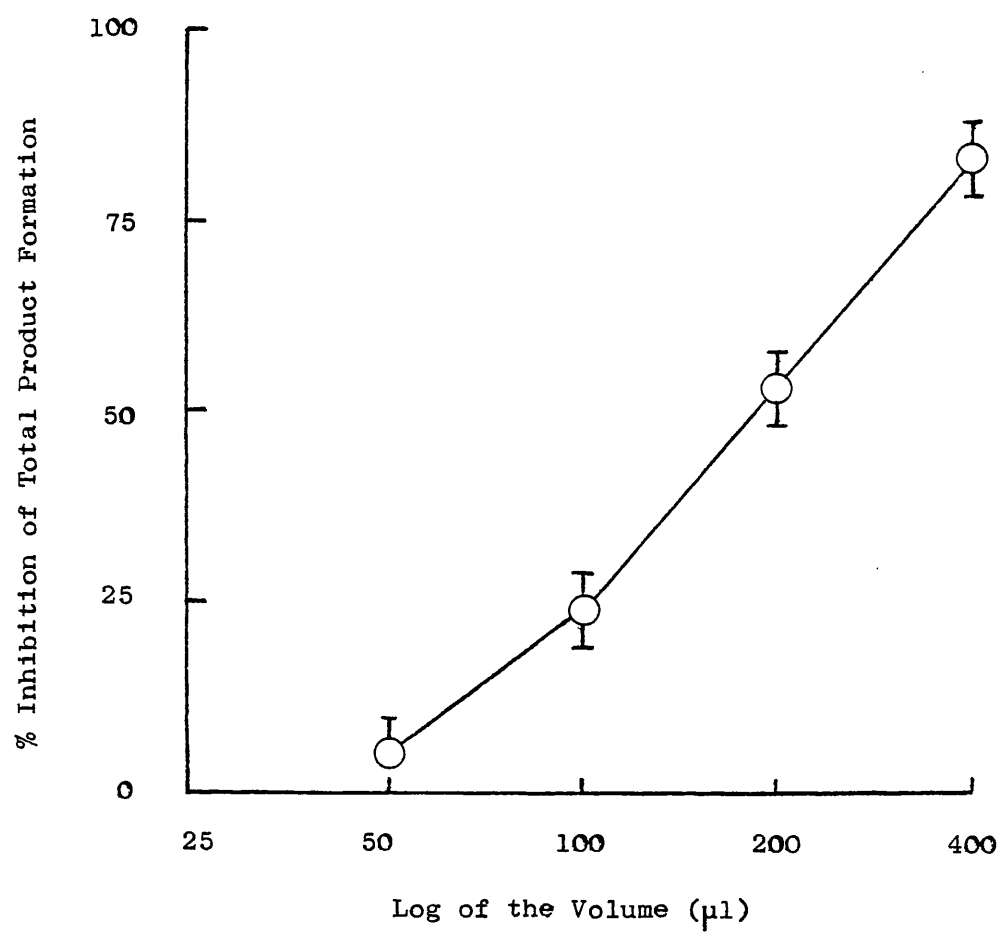
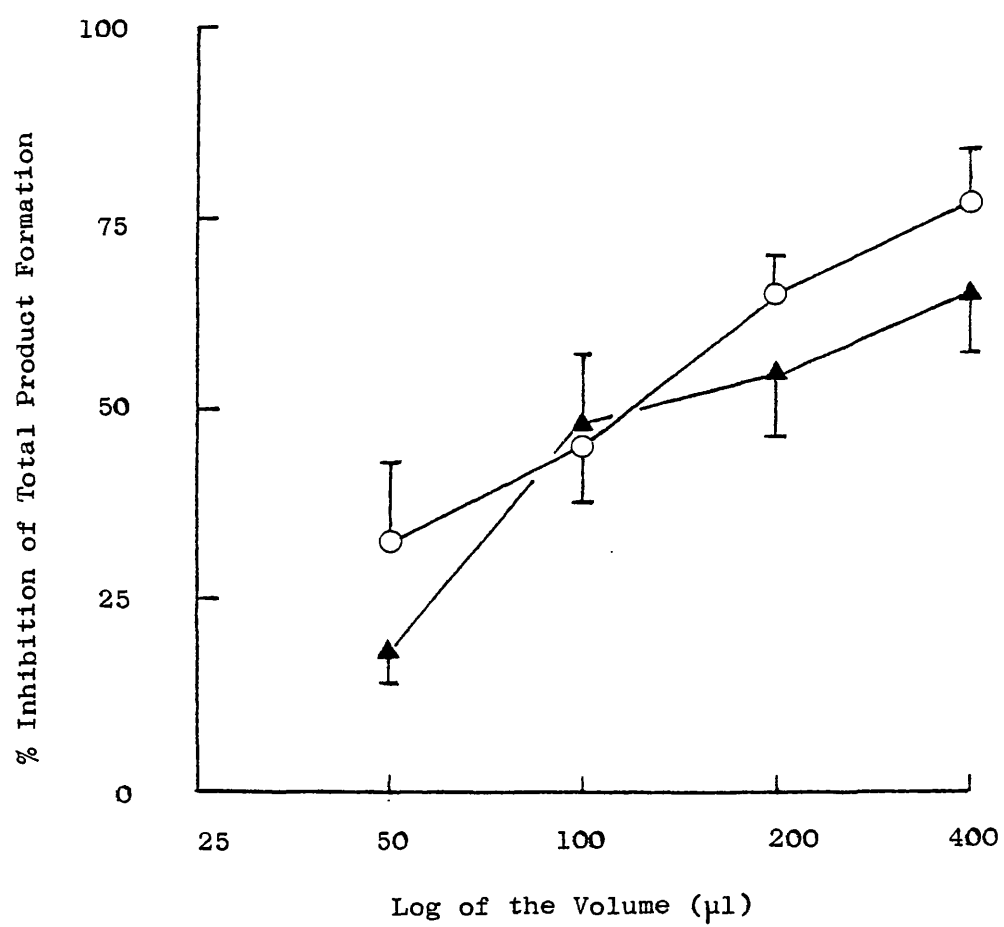


Figure 34. The inhibition of decidual (O-O) and myometrial (▲-▲) PG synthetase by placental microsomal supernatant during a 30 minute incubation.



To establish that the inhibition was not due simply to a dilution of the $[1-^{14}\text{C}]$ -AA with endogenous AA present in the supernatant; the supernatants were passed through a short column of silicic acid (8 x 0.8 cm) under nitrogen with a small quantity of $[1-^{14}\text{C}]$ -AA as a marker. This showed that all but $10.2 \pm 1.8\%$ ($n = 11$) of the labelled AA was retained on the column. This should have greatly reduced the quantity of endogenous AA present in the supernatants, but there was no noticeable effect on the inhibitor activity. All supernatants were subsequently passed through silicic acid columns before being added to incubates.

Since the decidual, myometrial and placental microsomal supernatants prepared from rats on day 22 of pregnancy it was decided to try the myometrial microsomal supernatant from non-pregnant rats (there was insufficient decidual tissue for a similar decidual supernatant preparation). The results shown in Figure 35 revealed that the non-pregnant myometrial supernatant had some inhibitory activity on PG synthesis by both myometrial and decidual microsomes. With 400 μl of the supernatant there was a 40% and 65% inhibition of PG synthesis by myometrial and decidual microsomes respectively, the myometrium appearing to be far less affected by this supernatant preparation.

3.10 Inhibition of PG Synthesis by Placental Microsomal Supernatant

Owing to the greater quantity of placental microsomal supernatant available this was chosen as the primary source of material for the continuation of this study. The effect of 400 μl of placental microsomal supernatant on AA metabolism by myometrial and decidual microsomes is illustrated in Figures 36 and 37. It can be seen that PG synthesis is greatly reduced when compared to control

Figure 35. Inhibition of decidual (O-O) and myometrial (▲-▲)
microsomal PG synthesis by non-pregnant myometrial
microsomal supernatant.

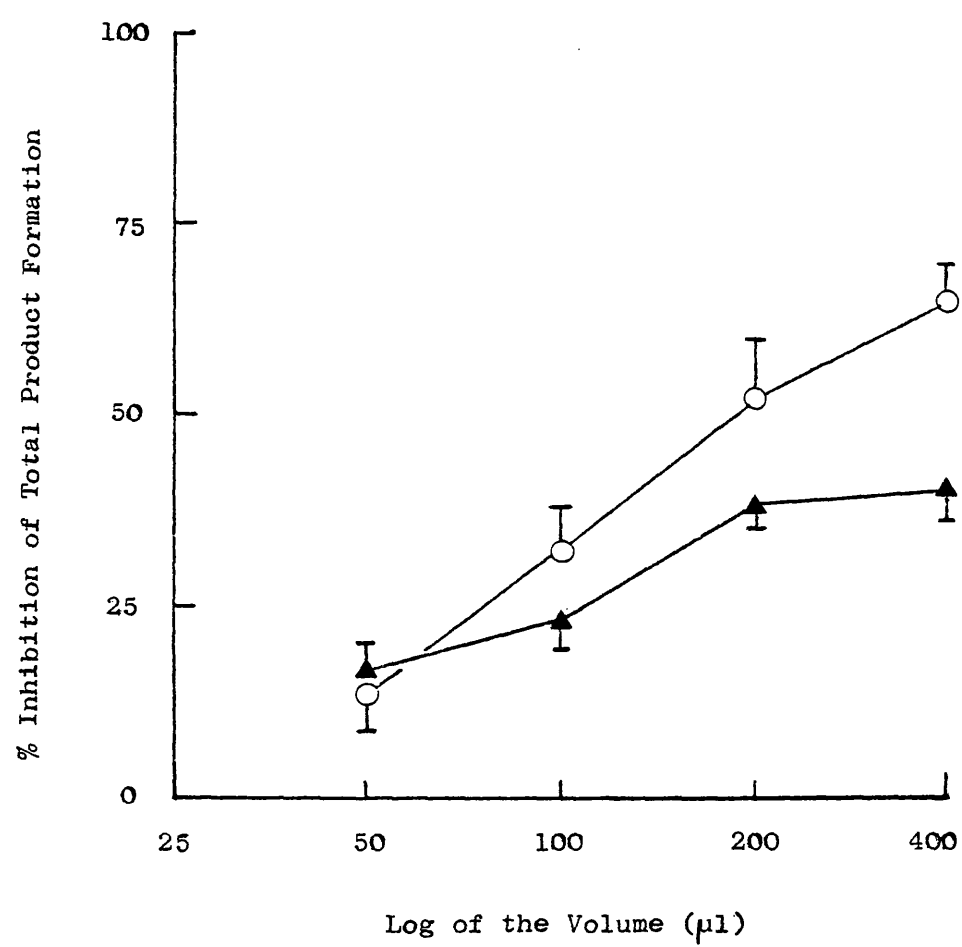


Figure 36.. The effect on AA metabolism by myometrial microsomes when incubated with (1) 400 μ l of placental microsomes supernatant (lower TLC), (2) 400 μ l of boiled placental microsomal supernatant (upper TLC). TLC plates were developed in solvent system 1 (chloroform/methanol/acetic acid/water, 90:9:1:0.65).

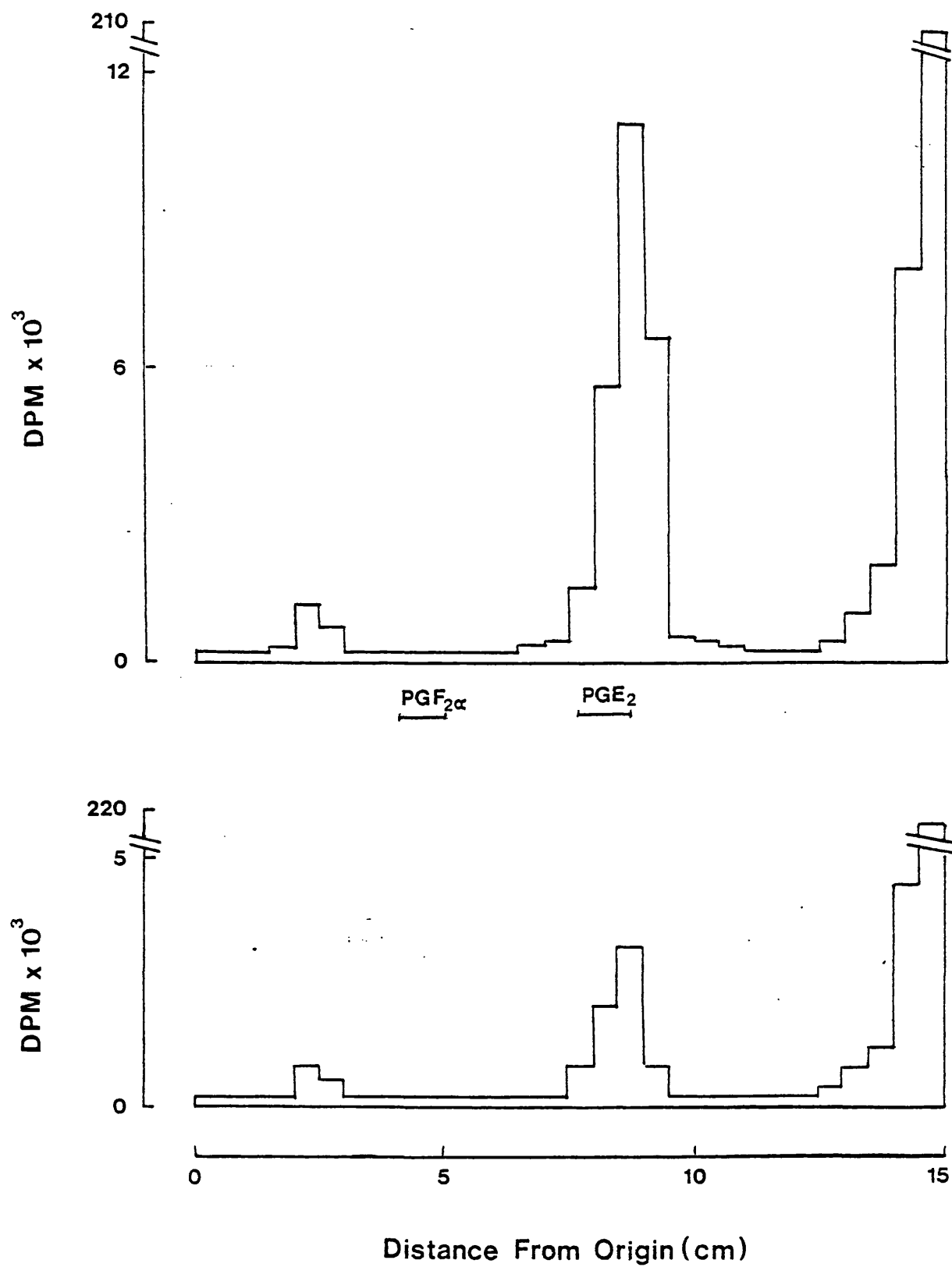
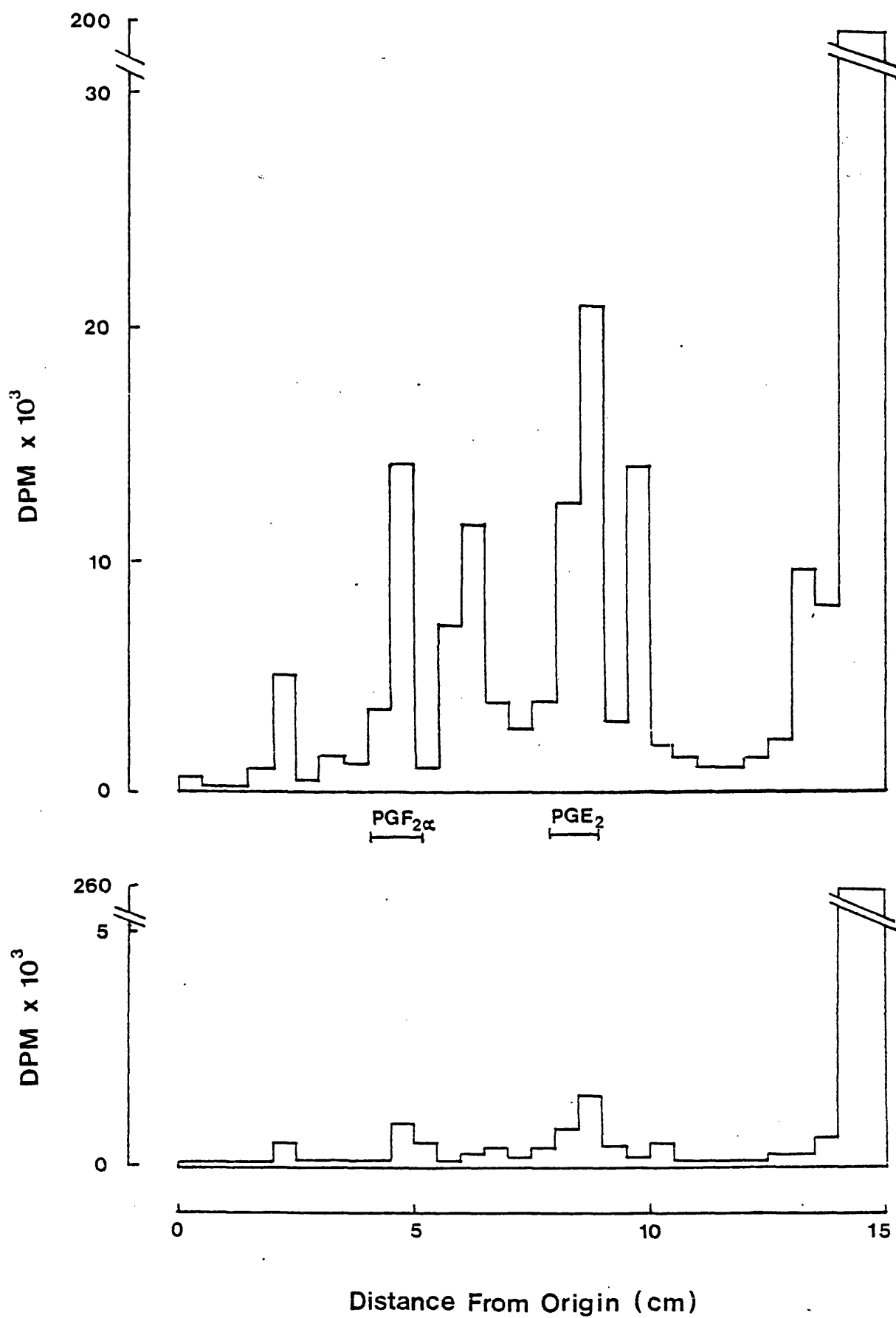


Figure 37 The effect on PG synthesis by incubating decidual microsomal supernatant (lower TLC) compared to a standard incubation plus 400 μ l of boiled placental microsomal supernatant (upper TLC). TLCs were developed in solvent system 1 (chloroform/methanol/acetic acid/water, 90:9:1:0.65).



incubations with 400 μ l of boiled placental microsomal supernatant. It was found that apart from boiling, the inhibitory activity of the placental microsomal supernatant disappeared if stored overnight at room temperature, 0°C, or -20°C. A lyophylate was therefore successfully prepared for long term storage and ready availability of the supernatant. The supernatant was lyophilised in a variety of buffers, phosphate buffer (pH 8.0 and pH 7.4), Krebs and tris (0.1M, pH 8.0). None of these buffers appeared to affect the activity of the reconstituted supernatant. Tris buffer was regularly used as it was nearer the buffer the uterine microsomes were incubated in. Controls incubated with the required volume of tris buffer (50-400 μ l) showed that this addition had little effect on the PG production by uterine microsomes. The final volume was kept at 2.0 ml for all incubations. A mean concentration for the lyophylate in tris buffer was 15.3 ± 1.8 mg/ml, ($n = 5$) calculated from the original volume, with a mean protein concentration of 4.26 ± 0.76 mg/ml, ($n = 5$). When it was used it was resuspended to the original microsomal supernatant volume.

The lyophilised placental supernatant was checked for inhibitory activity against ram seminal vesicle microsomes (RSVM). Initially using the thiobarbiturate assay, but the red colouration of the placental microsomal supernatant interfered with this. The radio-metric assay was, however suitable. Control synthesis of PGs by the RSVM was $60.9 \pm 2.0\%$. when the RSVM were incubated with 400 μ l of the reconstituted placental supernatant (in a final volume of 2 ml) $41.2 \pm 4.2\%$ ($n = 8$) of the $[1-^{14}\text{C}]$ - AA was converted to PGs. This was a significant decrease, $p < 0.001$ and indicated a 32% inhibition of RSVM.

The placental microsomal supernatant was also incubated with tritiated $\text{PGF}_{2\alpha}$ and PGE_1 to check that there was no degradation of freshly synthesized PGs. This procedure produced no evidence of metabolites of PGs, 94% and 97% of the radioactivity recovered had identical R_fs to tritiated $\text{PGF}_{2\alpha}$ and PGE_1 applied directly to the TLC plate. To gain an idea of the log dose relationship of the placental microsomal lyophilates one batch was prepared in distilled water (pH 8.0) and a percentage inhibition v log dose graph constructed using the dry weight of the lyophilate (Fig. 38). This was similar to figure 34, where percentage inhibition was plotted against the log of the volume of placental microsomal supernatant, however, in this case the myometrial microsomes were much more affected than decidual microsomes, 2 mg of the lyophilate inhibited myometrial PG synthesis by 66%, but decidual PG synthesis by only 29%.

The effect of the placental supernatant on the proportions of PGs synthesized by the decidual and myometrial microsomes was examined (Table 11). This revealed there was no significant difference in the proportions of any of the AA metabolites synthesized by the decidual and myometrial microsomes when they were incubated with any volume of placental microsomal supernatant between 50-400 μl .

Figure 38. The inhibition of decidual (O-O) and myometrial (▲-▲) microsomeal PG synthesis by a lyophylised preparation of placental supernatant, lyophylised in distilled water.

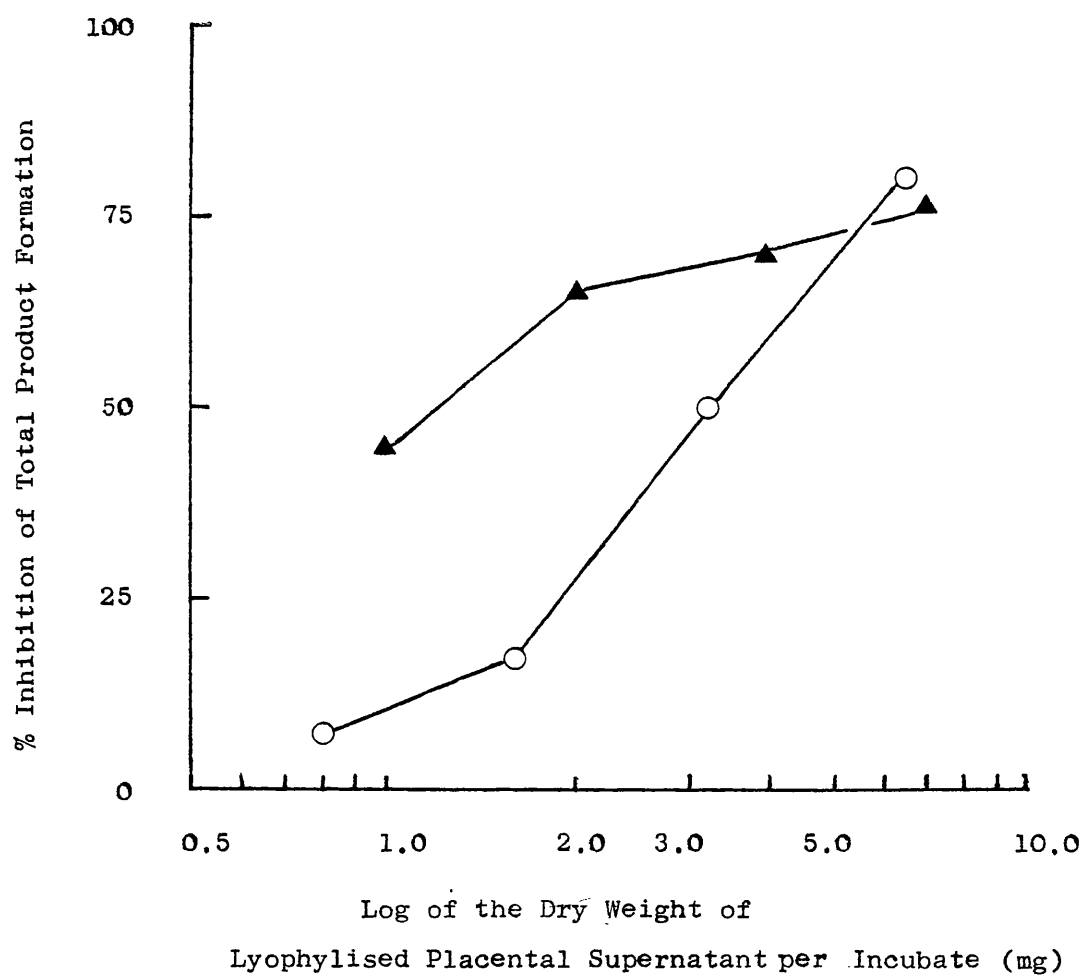


Table 11 Proportions of PG-like products produced by decidua and myometrial microsomes
when inhibited by Placental Microsomal Supernatant

Volume Placental Supernatant Added to Incubate (μl)	D E C I D U A					M Y O M E T R I U M	
	% COMPOSITION OF PRODUCTS					% COMPOSITION OF PRODUCTS	
	PGF _{2α}	TXB ₂	PGE ₂	6-oxo-PGF _{1α}	PGD ₂	PGE ₂	6-oxo-PGF _{1α}
0	19.9±2.5	13.9±2.6	32.8±1.7	14.4±1.7	7.6±2.0	9.0±0.8	86.9±0.7
50	19.9±4.5	11.1±1.2	34.5±4.0	18.0±2.2	10.7±2.1	7.3±1.2	82.7±0.6
100	17.3±0.8	10.9±1.1	35.2±2.2	16.4±1.7	8.7±1.6	10.1±2.9	80.9±7.7
200	17.1±1.0	12.9±2.5	30.7±2.9	13.9±0.9	8.4±1.8	10.6±3.7	81.3±5.9
400	20.0±0.7	12.1±2.1	34.8±2.1	16.8±1.3	8.2±0.6	8.2±0.4	83.2±0.1

CHAPTER FOUR

DISCUSSION

Arachidonic Acid Metabolism by Uterine Microsomes

The isolated pregnant rat uterus has been shown to release PGs into the bathing fluid, and this release increases as parturition approaches (Vane and Williams, 1973). Further it was found that cell free homogenates of decidua and myometrium were capable of synthesizing PGs from exogenous AA, with the majority of activity being found in decidual homogenates (Williams, Sneddon and Harney, 1974). Therefore in this study microsomes of decidual and myometrium tissue taken on day 22 (day of delivery) were studied. The exact location of the PG synthetase within the microsomal pellet was not investigated. However, Bohman and Larsson (1975) using a rabbit renal medullary microsome preparation found the majority of PG synthetase activity associated with the cytomembrane fraction, which consists of closed vesicles of smooth and ribosomal studded membranes. They concluded that the PG synthetase is located in the endoplasmic reticulum of renal medullary cells.

This study confirmed that the decidua has a more active PG synthetase than that of the myometrium, metabolizing three times more AA than the myometrium in a 30 minute incubation. It has been established by TLC techniques that the products of the myometrial PG synthetase is mainly 6-oxo-PGF_{1α} (77%) accompanied by a small proportion of PGE₂ (10.5%). However, the PG synthetase in the decidual microsomes synthesizes a wider variety of products; the major product being PGE₂ (37.4%), followed by PGF_{2α} (17.8%), 6-oxo-PGF_{1α} (15%), TXB₂ (12.8%) and PGD₂ (10.7%). These are the products detected and values obtained using TLC techniques. GC/MS analysis confirmed these findings except for the presence of PGD₂. The lack of the major ions associated with PGD₂ was unclear. However, analysis was only conducted for one experiment, PGD₂ was consistently isolated by chromatographic procedures.

Attempts were made to establish the presence of PGE_2 in the products of microsomal incubations by chemical conversion. Products of microsomal incubations which had Rf values corresponding to authentic PGE_2 were treated with sodium hydroxide or sodium borohydride, such treatment converts PGE_2 to PGB_2 or a mixture of $\text{PGF}_{2\alpha}$ and $\text{PGF}_{2\beta}$ respectively (Sih and Takeguchi, 1973). While these conversions were successful much of the original labelled product did not extract from the acidified aqueous phase. As later experiments revealed that peak C was a mixture of PGE_2 and 6-oxo- $\text{PGF}_{1\alpha}$ this may have accounted for this unexpected observation. Indeed authentic 6-oxo- $\text{PGF}_{1\alpha}$ was found to be unaffected by sodium hydroxide. However, it was reduced by sodium borohydride treatment. This is of interest as Pace-Asciak (1976) found that if the same reaction was carried out in methanol at room temperature 6-oxo- $\text{PGF}_{1\alpha}$ was found to be unreactive. However, in these studies water at 37°C was used as the solvent and it is probable that was facilitated by this, water having a well known hydrogen bonding ability and able to stabilise both cations and anions more effectively than methanol.

Incubation Conditions

When investigating influence of pH on PG production maximum conversion occurred at pH 8.0 for both decidua and myometrial microsomes. Although this is slightly alkaline many other microsomal tissues have been shown to have a pH optima close to this value, i.e. sheep seminal vesicle microsomes. pH 8.0, (Wallach and Daniels, 1971); bovine seminal vesicle microsomes pH 7.8-8.2, (Flower, Cheung and Cushman, 1973); rheumatoid synovial microsomes pH 8.0 (Crook and Collins, 1975), and reproductive tract microsomes of the house cricket pH 8.0 (Deslephano,

Brady and Woodall, 1976). Rabbit kidney medulla microsomes have been shown to have pH optima between pH 7.5-8.3 (Blackwell, Flower and Vane, 1975b; Tai, Tai and Hollander, 1976; Schwartzman, Gafni and Raz, 1976). For the uterine microsomes it was found there was a considerable decrease in substrate conversion as the pH decreased and if the assay had been carried out at physiological pH (i.e. 7.4) then there would have been a 30% reduction in substrate conversion. This sharp decrease in substrate conversion due to a small alteration in pH has also been found in many of the above mentioned preparations. pH is known to have an effect on the ionisation of the substrate, its binding to the enzymic active site and on the binding of prosthetic groups, and a combination of these effects may be responsible for the influence of pH observed.

In investigating the time course of AA metabolism it was noticeable how rapidly substrate conversion proceeded in the first five minutes after the initiation of the reaction. Thereafter although substrate conversion continued it took 25 minutes to double the amount of product formed for both decidual and myometrial microsomes. This decrease in product formation after five minutes supports the work of Wallach and Daniels (1971) who found that PG synthesis by sheep seminal vesicle microsomes was only linear with respect to time for the first 6 minutes, thereafter the rate declining. They suggested that one or more reaction products of a fatty acid nature inhibited the enzyme and they claim it is a competitive inhibition as the enzyme can be reactivated if the microsomes were separated by centrifugation and added to fresh buffer. Uterine horns from non-pregnant rats have also been shown to produce maximal amounts of products in the first five minutes,

which then declined, production was restarted if the horn was transferred to fresh buffer (Ishikawa and Fuchs, 1978). They also concluded this inhibition was due to the accumulation of an inhibitor in the incubation medium.

It was demonstrated that there was no linear relationship between protein concentration of microsomes and product formation. Very similar results were obtained by Wlodawer, Kindahl and Hamberg (1976) using microsomes of cow and quinea-pig non-pregnant uteri. They failed to correlate concentration of microsomes with quantity of PGs synthesized. This inhibition may be related to the reduction of PG synthesis as incubation time progresses.

When the influence of substrate concentration on PG synthesis by decidual and myometrial microsomes was investigated it was found that the PG synthetase complexes did not conform to classical enzyme kinetics. With decidual microsomes there was a marked inhibition of PG synthesis with increasing substrate concentration. A similar inhibition also occurred in myometrial microsomes but was less well defined and was at a lower concentration. Substrate inhibition of PG synthesis has also been noted in bovine seminal vesicle microsomes (Yoshimoto, Ito and Tomita, 1970; Duvivier, Wolf and Heusghem, 1975) and rabbit kidney medulla microsomes (Schwartzman, Gafni and Raz, 1976). It has been suggested that substrate inhibition by fatty acids (such as AA) is caused by the detergent properties exerted by high concentrations of the fatty acids (Pande and Mead, 1968). However, this does not account for inhibition of the two uterine microsomal preparations occurring at different substrate concentrations. The common postulate for substrate inhibition is that the active site of the enzyme is crowded with substrate molecules competing to occupy it, so that no

particular molecule can combine with all the binding groups of the active site. The result is that the binding groups are split between two or more substrate molecules. These multi-substrate complexes are rarely active as the substrate is usually not properly orientated (Walter, 1965). Substrate inhibition is a fairly common phenomenon, other examples are hydrolysis of urea by urease (Laidler and Hoare, 1949), hydrolysis of carbobenzoxyglycyl-L-tryptophan by carboxypeptidase (Lumry, Smith and Glanz, 1951), and the reaction of ethanol and NAD^+ catalysed by alcohol dehydrogenase, ethanol being the inhibitor. (Harnes, Bronskill, Gurr and Wong, 1972). As the PG synthetase present in decidual and myometrial microsomes was not a "classical" enzyme it was not possible to calculate the K_m or V_m of these enzyme complexes.

At the time the preparation of an acetone-pentane powder was undertaken, the complete dependence of the PG synthetase in the decidual and myometrial microsomal preparations on hydroquinone was unknown. It is thought this contributed towards the unsuccessful result of this work, as hydroquinone was not used in the buffer while preparing the powder. A stable lyophylate of uterine PG synthetase from both decidual and myometrial microsomes using normal buffer containing hydroquinone has recently been prepared (Harrowing, personal communication).

Cofactors

It is notable that crude uterine homogenates synthesize predominantly $\text{PGF}_{2\alpha}$ as estimated by bioassay (Vane and Williams, 1973). However, 6-oxo- $\text{PGF}_{1\alpha}$ has been shown to be the main product in homogenates of the pseudopregnant rat uterus (Fenwick, Jones, Naylor, Poyser and Wilson, 1977). $\text{PGF}_{2\alpha}$, PGE_2 and PGD_2 were also detected

in the pseudopregnant rat uteri homogenates by Fenwick et al (1977). More recently in isolated pregnant rat uteri at term Phillips and Poyser (1979) found PGD_2 , 6-oxo-PGF $_{1\alpha}$ and TXB $_2$ to be the major PG-like materials found with smaller quantities of PGE $_2$ and PGF $_{2\alpha}$. There is then some agreement between the products produced by the microsomal preparation of rat uteri and other rat uteri preparations. The different proportions of products seen may possibly be due to the inevitable replacement of the natural cofactor by exogenous cofactors which may differentially activate components of the PG synthetase.

When investigating the influence of cofactors on PG-like product formation by decidual and myometrial microsomal suspensions the presence of hydroquinone in the buffer before resuspension of the microsomes was found to be essential for AA metabolism. This is a major difference from other PG synthetase preparations such as RSVM and kidney medulla microsomes which retain a high synthetic capacity in the absence of hydroquinone (Cottee et al, 1977; Yoshimoto et al, 1970). In non-pregnant uterine microsomes of cow and guinea-pig Wlodawer et al, (1976) found hydroquinone to be inhibitory in any concentration. The role of hydroquinone in PG synthesis is as yet undertermined. Pace-Asciak (1972) postulated that the anti-oxidant cofactors (e.g. noradrenaline and adrenaline) stimulate the PG synthetase complex by stabilising the enzyme in the active form. Another postulate is that hydroquinone, or a similar cofactor is required for the attachment of oxygen to the fatty acid (Yoshimoto et al, 1970). Smith and Lands (1971) postulated that phenolic cofactors are required to activate the PG synthetase before the initial oxygenation reaction. The simplest explanation for the above postulates is that hydroquinone is responsible for the

reduction of one or more of the reactive groups at the active site of the cyclo-oxygenase component of the PG synthetase complex. The ability to continually reduce the vital group(s) at the active site may decrease with time and be due to insufficient hydroquinone as time progressed in a reduced form. However, it must be noted that increasing the concentration of hydroquinone above the optima does not increase the rate of PG synthesis, but inhibits it. So the action must be more complex, possibly by group(s) at the active site binding minor reaction products.

The inclusion of GSH, or L-cysteine although increasing PG synthesis were not essential for PG synthesis. If both were absent then there was only a third of the synthetic activity when both were present. L-cysteine is not commonly used as a cofactor. However, GSH has a longstanding involvement with PG synthesis. It has been reported to stimulate PG biosynthesis by bovine seminal vesicles (Yoshimoto et al, 1970), sheep seminal vesicle microsomes (Samuelsson, 1970), rat skin microsomes (Kingston and Greaves, 1976) and in several microsomal preparations of rabbit tissues (Tai, Tai and Hollander, 1976; Pong and Levine, 1976). There are two predominant postulates on the action of GSH. The first suggests that GSH is required for the reduction of the intermediate endoperoxides (Yoshimoto et al, 1970). Others also present evidence that it may specifically activate the endoperoxide isomerase component to catalyse the formation of PGE_2 , (Lands, Lee and Smith, 1971; Tai, 1976). The second postulate propounds that the function of the GSH is to cycle the electron donor (e.g. hydroquinone) back into the reduced state (Flower, Cheung and Cushman, 1973; Foss, Takeguchi, Tai, and Sih, 1971). It was shown that unlike some PG synthetase complexes the proportions of the different products synthesized by

decidual and myometrial microsomes were unaffected by the inclusion of thiol containing cofactors. This is unlike RSVM (Cottee et al, 1977), rat skin microsomes (Kingston and Greaves, 1976) and bovine seminal vesicle microsomes (Foss et al, 1971), where inclusion of GSH resulted in increased PGE_2 synthesis at the expense of the other products. Also taking into account that hydroquinone in thiol-free buffer oxidises spontaneously (within 60 minutes at 0°C). The probable mechanism employed by the thiol containing cofactors to stimulate both decidual and myometrial microsomal PG synthesis is the reduction of the anti-oxidant supplying the synthetase with fresh reducing equivalents.

The finding that EDTA increases PG synthesis by decidual and myometrial microsomes is in line with the findings of other workers (Ziboh, 1973; Pace-Asciak, Morawska, Coceani and Wolfe, 1968). This stimulation of PG synthesis is thought to be caused by the EDTA chelating metal ions such as zinc, cadmium and copper which have been shown to be inhibitory to PG synthetase (Nugteren et al, 1966). An alternative but less likely possibility is that the EDTA is chelating calcium. This would prevent phospholipase A_2 located in the microsomal fraction (Luckner and Renz, 1975) from liberating AA from phospholipid present and diluting the radiolabelled AA (Bohman and Larsson, 1975). The phospholipase in the microsomal fraction is calcium dependent (Newkirk and Waite, 1973). However, there is evidence that the phospholipase A_2 in human decidua and myometrium, possibly responsible for the release of uterine PGs, is contained in lysosomes, and is calcium independent (Akeson, 1975).

Effects of Cofactors

In incubations using decidual and myometrial microsomal suspensions it was possible to replace hydroquinone with other anti-oxidants as is the case with other tissue homogenates (Sih, Takeguchi and Foss, 1970; Pace-Asciak, 1972; Sykes and Maddox, 1972). Of the cofactors tried it was found that histamine and L-tryptophan plus haemoglobin were poor replacements for hydroquinone although L-tryptophan and haemoglobin have been reported to effectively replace hydroquinone using bovine seminal vesicles (Miyamoto *et al*, 1974). This suggests that in the uterus at least L-tryptophan is not the endogenous cofactor, as it had been postulated to be for bovine seminal vesicular gland (Chan, Nagasawa, Takeguchi and Sih, 1975). Histamine was also found to be a poor cofactor for tumour PG synthetase by Sykes and Maddox (1972). 5-Hydroxytryptamine was found to be the best replacement cofactor in decidual incubations producing a substrate conversion similar to that seen with hydroquinone. Adrenaline and noradrenaline were approximately half as effective as 5-hydroxytryptamine. A different pattern was seen with myometrial microsomes where 5-hydroxytryptamine, noradrenaline and adrenaline all proved to effect a higher substrate conversion than did hydroquinone (although only noradrenaline caused a significant increase).

When comparing the molecular structure of the different molecules it is notable that the poor cofactors histamine and L-tryptophan possess no phenolic hydroxyl group while the effective cofactors had at least one phenolic OH group, Suggesting this group is a prerequisite for cofactor function. L-Ascorbic acid was included to stabilise the buffers containing adrenaline and noradrenaline. When used as a replacement for hydroquinone in decidual microsomal

incubations it proved more effective than histamine and one quarter as effective as hydroquinone. With myometrial microsomes it was approximately 0.75 times as effective as hydroquinone in stimulating PG synthesis. These results are perhaps not surprising as L-ascorbic acid is an anti-oxidant as is hydroquinone. But it may indicate that the myometrial PG synthetase is less selective in its cofactor requirements. However, there is evidence that in solution L-ascorbic acid is cyclic and has free hydroxyl groups which may provide some structural similarity with the phenolic biogenic amines which have been mentioned. As L-ascorbic acid proved so effective in stimulating AA metabolism by myometrial microsomes it is possible that the high synthesis seen using adrenaline and noradrenaline could be due to the fact that the buffer also contained L-ascorbic acid as an anti-oxidant. However, when L-ascorbic acid was included in incubations along with hydroquinone as the cofactor the L-ascorbic acid did not promote a further increase in PG synthesis over that seen with hydroquinone alone. This suggests that the high stimulation seen in myometrial AA metabolism using adrenaline and noradrenaline as cofactors is actually due to the presence of these compounds and not due to interference from L-ascorbic acid.

Apart from affecting overall product formation the substituted cofactors also affected the relative proportions of the products which were formed by the decidual microsomes. All the cofactors used to replace hydroquinone caused a significant increase in $\text{PGF}_{2\alpha}$. While 5-hydroxytryptamine and histamine caused a significant decrease in TXB_2 synthesis. In contrast there was no significant effect on the relative proportions of 6-oxo- $\text{PGF}_{1\alpha}$ PGE_2 synthesized by the myometrial microsomes. It has been found by Sykes and Maddox (1972) using tumour cell homogenates, and Sih et al (1970) using

bovine seminal vesicle microsomes that adrenaline and 5-hydroxy-tryptamine favoured the synthesis of PGF more than hydroquinone.

The presence of an endogenous cofactor may explain why PGF_{2α} is produced in smaller quantities in the microsomal suspension than in crude homogenates (Vane and Williams, 1973). There is no clear indication from this data whether one of the cofactors investigated is the natural cofactor. The catecholamines have been favoured in this role by Pace-Asciak (1972) and Sih et al (1970). This appears to be a real possibility in the uterus. The uterus has been shown to contain adrenaline and noradrenaline stored in chromaffin cells, which are independent of the nervous supply. 80% of the stored catecholamines is depleted soon after the onset of labour (Wurtman, Axelrod and Potter, 1964; Barnea and Schelesnyak, 1965). The fact that adrenaline and noradrenaline are not the best cofactors available for the decidual PG synthetase need not necessarily be important. The myometrium constitutes 80% of the uterus, the decidua only 4% (with the other 16% being myometrium lying under the placenta). Even though the decidua is more active than the myometrium it could only be equivalent to 15% of the myometrial contribution which must therefore supply the largest quantity of PGs. It may be that it is the type rather than the quantities of PGs synthesized by the decidua which is important.

Influence of Gestation on Uterine PG Synthesis

Studies on uterine PG synthesis during the early stages of pregnancy are hampered by two factors, firstly the small size of the uterus and hence the presence of an extremely small quantity of decidual tissue. Secondly, the low PG synthetic capacity of the

uterus at this time. Thus investigations were limited to days 13-22 of pregnancy. Non-pregnant myometrium was used for comparison but again endometrial tissue was not investigated due to the small amounts present.

In both decidual and myometrial microsomes PG synthetic capacity increased as parturition approached. From days 14-19 the myometrium produced considerably more PGs than the decidua while on days 20 and 21 their synthetic capacity was approximately the same. Maximum PG synthesis occurred on day 22 (expected day of delivery), with the decidual synthetic capacity more than double that of the myometrium which appeared to plateau on days 19-22. The synthetic capacity of both microsomal preparations was considerably diminished one day post-partum.

What are the factors which account for this change in PG synthesizing capacity in the uterus? Uterine weight trebles between days 13 and 22 and it could be argued that although the total synthesizing capacity of the uterus alters, the activity per unit weight of remains the same. However, this is unlikely to account for the changes which were seen because between days 21 and 22 of pregnancy uterine weight remains fairly constant but over this period there is a massive increase in decidual PG synthesizing capability. The changes seen would seem to indicate that there is a real increase in the activity of the PG synthetase complex in the uterus. Such studies do not preclude the possibility that in the whole uterus the availability of AA may be the limiting factor in PG synthesis. However, as microsomal preparations utilising exogenous substrate were used, no information was gained on this point.

The changes observed may have been produced by the marked fluctuations in the plasma levels of sex hormones which occur towards the end of gestation. Plasma progesterone levels in the rat rise sharply before implantation and remain elevated throughout most of gestation but decline sharply before parturition (see Fuchs, 1978). However, oestrogen levels are low during gestation but rise sharply just before parturition (Fuchs, 1978). Data available suggests that the actions of these two hormones on the uterus may be antagonistic. For instance dosing pregnant sheep with progesterone delays parturition for as long as dosing is continued (Liggins et al, 1972), while in the pregnant rat there is little change in uterine PG output when dosed with progesterone. (Harney, Sneddon and Williams, 1974). The only direct effect of progesterone on PG synthesis in the uterus of goats and sheep is that progesterone withdrawal precedes the rise in PG levels in uterine venous plasma (Thorburn et al, 1972) and that progesterone has been shown to suppress the release of $\text{PGF}_{2\alpha}$ into uterine venous blood in sheep although tissue concentrations rise in a normal fashion (Mitchell and Flint, 1977). While, Ham et al, (1975) and Sharma and Garg (1977) found that the PG content of the non-pregnant uterus in ovariectomised rats could be increased by dosing with oestrogen, and that the PGE/PGF ratio was also altered. This increase was abolished if the oestrogen was co-administered with progesterone (Ham et al, 1975). Fenwick et al (1977) found that giving Tamoxifen (an anti-oestrogenic drug) to pregnant rats decreased the ability of uterine homogenates to synthesize PGs. The above evidence indicates that oestrogen acts to stimulate uterine contractility and PG synthesis whereas progesterone may act as a brake on this process ("progesterone block" - Csapo,

1956, 1975). The decline in progesterone levels at term allows oestrogen to 'activate' the PG synthetase and may influence the ratio of products synthesized. The stimulant effect of oestrogen on uterine PG synthesis is probably brought about by its action in increasing protein synthesis and hence uterine content of the synthetase enzyme (see Means and O'Malley, 1972). It is also possible that the increase in PG levels could be due to a decrease in PG catabolism as this can be influenced by steroids (Blackwell and Flower, 1976). However, in this study microsomal suspensions were used which had been demonstrated to be free of PG catabolising enzymes, and any PGs formed during preparation of the microsomes would be lost in the supernatant. This must support a real increase in PG synthetic capacity.

The proportions of PG-like products synthesized by the myometrial microsomes were unchanged as pregnancy progressed, 6-oxo-PGF_{1α} being the major product throughout. In contrast it was found that the proportions of products synthesized by decidual microsomes altered as parturition approached. Between days 13 and 20 6-oxo-PF_{1α} was the major decidual PG synthesized. PGE₂ became the major AA metabolite only on day 21-22 of pregnancy. Despite the fact that the synthetic capacity of the myometrium is only a third of that of the decidua, because there is so much more of it, 6-oxo-PGF_{1α} is the major PG synthesized by the uterine microsomes.

The Inhibition of Decidual and Myometrial PG Synthesis by NSAIDs.

The results indicated that microsomal preparations of uterine PG synthetase are susceptible to inhibition by NSAIDs. This is not exceptional as NSAIDs have been shown to be potent inhibition of PG synthesis in many systems (Flower, 1974; Pong and Levine, 1976). In general the I.D.₅₀ values (that dose of NSAIDs which cause a 50% inhibition of PG synthesis) were lower for the myometrial microsomes than for the decidual microsomes. This difference is not surprising in view of the fact that the decidual microsomes have three times more synthetic activity than the myometrial microsomes. However, the difference is not as great as might be expected with the difference in synthetic capacity of the different microsomal preparation. There are several possible reasons for this. Firstly, substrate concentration can affect the I.D.₅₀ for particular inhibitor e.g. aspirin and indomethacin have a greater potency at low substrate concentrations compared to high concentrations (Cushman and Cheung, 1976). This is infact seen with the microsomal preparations used. With the myometrial PG synthetase the substrate concentration used was maximal whereas the decidual PG synthetase utilised a similar substrate concentration which was considerably below its maximum. These differences may have artificially reduced the differences between the two I.D.₅₀ values. Therefore, it is not really valid to compare actual I.D._{50s} obtained for the same drug using different synthetase preparations but it is still valid to compare the order of potency. The I.D.₅₀ values obtained for indomethacin and aspirin are comparable to those obtained with microsomes from rabbit kidney (Flower, 1974) and non-pregnant rabbit uterus (Pong and Levine, 1976). Aiken (1974) examined a number of NSAIDs for effects on motility of the rat pregnant uterus

in vitro (where motility is related to PG output). From his results aspirin has a similar I.D.₅₀ to that obtained in the present studies but indomethacin was found to be more potent. This may be a further reflection of the influence of substrate concentration, as using uterine strips PG synthesis is dependent on endogenous substrate concentrations.

The relative potency of drugs was expressed relative to TYA because TYA is an acetylated substrate analogue of AA and therefore the closest inhibitor in structure to A.A. It reacts with the active site of the cyclo-oxygenase (Downing, Ahern and Bachta, 1970) and its inhibitory activity consists of an initial concentration-dependent effect (Ahern and Downing, 1970) and a time-dependent irreversible inhibition (Lands, Le Tellier, Rome and Vanderhoek, 1973) similar to many NSAIDs (Flower, 1974). The rank order of potency revealed that flurbiprofen was the most potent inhibitor studied. This finding is in agreement with that of Crook and Collins (1976) who also found this drug most potent in inhibiting PG synthesis by microsomes of rheumatoid synovia. There was no difference in the rank order between the decidual and myometrial preparations.

Tranlycypromine has been included under the catch-all heading of NSAIDs though it is really a tricyclic anti-depressant. This drug has been shown to inhibit PG synthesis in cell free homogenates of guinea-pig lung (Lee, 1974) and to reduce motility of the pregnant rat uterus in vitro (Tothill, Bamford and Draper, 1971). Furthermore, tranlycypromine has recently been reported to be a selective inhibitor of prostacyclin synthetase (Gryglewski et al, 1976). When investigating the effects of inhibitors which act at more distal sites than the cyclo-oxygenase one may expect to see altered ratios of products under the influence of such drugs, as endoperoxides will still

be produced and would be free to be transformed by other enzymes such as isomerase, reductase or thromboxane synthetase. However, this was not seen. In the case of the decidua tranylcypromine inhibited production of all products to an equal extent, and rather surprisingly the I.D.₅₀ value for the myometrial microsomes was higher than that seen with the decidual preparation. Again a possible explanation of this discrepancy is that the activity of the prostacyclin synthetase in the myometrial preparation was higher than that in the decidua. This is certainly backed up by the amounts of 6-oxo-PGF_{1α} produced.

Metopirone is also a drug which is not an NSAID but an inhibitor of corticosteroid biosynthesis which has been reported to inhibit PG synthetase (Parnham and Sneddon, 1975). The drug was also reported to alter the ratios of PGs synthesized in uterine homogenates, PGE₂ formation being inhibited whereas an apparent increase in PGF_{2α} synthesis was noted. As the assay system used did not differentiate between PGE₂ and 6-oxo-PGF_{1α} it was possible that 6-oxo-PGF_{1α} synthesis was being selectively inhibited and allowing endoperoxides to be diverted for conversion by the reductase enzyme so increasing PGE₂ production. However, with decidual and myometrial PG synthetase metopirone was a very poor inhibitor and had no detectable effect on the ratio of products synthesized by either preparation. As the work of Parnham and Sneddon (1975) was carried out using crude homogenates it may be that metopirone did not act directly on the PG synthetase, that it requires a factor present in crude homogenates in order to exert its inhibitory effect and may only act at low concentrations of AA, as found endogenously.

Several previous studies have demonstrated in animals that it is possible to affect uterine motility with NSAIDs both in vitro and in vivo (Aiken, 1972; Aiken, 1974; Chester et al, 1972; Hertelendy, 1973; Novy et al, 1974; Vane and Williams, 1973). These results have clinical relevance because NSAIDs have subsequently been used to inhibit uterine contractions associated with premature labour (Zuckerman et al, 1974) and dysmenorrhoea (Lundstrom and Green, 1978). The isolated rat uterus in particular appears to provide a good model for studying the gross effects of these drugs (Williams et al, 1974). However, difficulties arise when trying to accurately assess the potencies of a variety of NSAIDs using this preparation (Lewis, Cottney and Sugrue, 1975). The use of a stable uterine microsome preparation overcomes these difficulties and allows accurate assessment of inhibitory potency. The fact that flurbiprofen is at least five times more potent than indomethacin suggests that this drug may be more useful to the obstetrician than indomethacin.

Microsomes of Placentae and MUP and the Inhibition of PG Synthesis by Microsomal Supernatants

The MUP was found to have a very low PG synthesizing capacity. This was surprising as the MUP is an integral part of the myometrium which as shown previously has considerable PG synthesizing capability. Since a microsomal preparation was used and exogenous substrate was added, the low synthetic capacity of the MUP could not be due to a lack of substrate. It was also found that placental microsomes prepared from placentae on day 22 of pregnancy were also a poor source of PG synthetase. This agrees with the findings of Carminati,

Luzzani, Saffientini and Lerner (1975) who used crude homogenates of rat placentae taken between days 8-21 of pregnancy. It is worth noting that human placental microsomes also have a low PG synthetic capability when taken at term (Duchesne, Thaler-Dao and Crastes de Paulet, 1978).

There has been no published explanation for this low PG synthetic activity. However, the low synthetic activity of the MUP and the placentae may be linked as they are in direct contact (decidua eroded in this region). One plausible hypothesis for such low synthesis is that there is an inhibitor of some type present in the placenta and that this inhibitor infiltrates into the MUP. In this respect it is of interest that the microsomal supernatants from decidual, myometrial and placental microsomes and non-pregnant rat myometrium contain a factor(s) which inhibited PG synthesis. This factor(s) was heat-labile and inhibited PG synthesis in a dose-dependant manner. The inhibitory activity could not be removed by passing the supernatants down silicic acid columns (which removed 90% of the labelled AA tracer). This indicates that the inhibition was not simply due to a dilution of labelled AA by endogenous substrate contained in the supernatant. In support of this was the finding that boiling AA for 1-2 minutes did not destroy the substrate and reduce subsequent PG synthesis by decidual and myometrial microsomes. Another possibility was that the uterine microsomal pellets contain substantial amounts of phospholipid and if phospholipase A_2 was present in the placental microsomal supernatant this would release endogenous AA. The phospholipase A_2 was originally thought to be membrane phospholipase and therefore, dependent on calcium ions, the presence of which should be minimised by the inclusion of EDTA in the buffer (Bohman and Larsson, 1975). However, in human decidua and myometrium

phospholipase A_2 is of lysosomal origin and is inhibited by calcium ions (Akesson, 1975). It is possible this is also present in rat decidua, myometrium and placenta, and would be found in the supernatant (which all contained inhibitory activity). This in addition to the resuspended microsomes the phospholipase A_2 in the supernatant would liberate endogenous substrate and so dilute the radiolabelled AA. This mechanism of inhibition is unlikely however, as uterine microsomes prepared in the presence of mepacrine, a phospholipase A_2 inhibitor did not affect inhibitory potency (Harrowing and Williams, 1979). In addition washing uterine microsomes with an acetone/pentane mixture, removed the phospholipid, still resulted in the same degree of inhibition as untreated microsomes. If release of endogenous substrate was responsible for the inhibitory effect, it would have been expected that the inhibitory potency of the supernatant would have decreased (Harrowing and Williams, 1979). The microsomal supernatant is also known to contain the enzymes which catabolise PGs. The probability that PGs synthesized were being metabolised by such enzymes which would evidence itself as an inhibition was eliminated by showing that incubation of the supernatants with radiolabelled PGE_1 or $PGF_{2\alpha}$ did not cause noticeable breakdown of these compounds.

The placental microsomal supernatant also inhibited AA metabolism by RSVM showing the inhibitory action was not restricted to uterine PG synthetase. The potency of the supernatant however, on RSVM was not as great as with uterine PG synthetase preparations. This result would be expected as the RSVM contains a more active synthetase.

The site of action of the placental inhibitor appears to be the cyclo-oxygenase, as all products of AA metabolism were reduced, with no change in the ratio of products synthesized.

Similar findings have been reported for the supernatants of several other tissues; bovine seminal vesicles (Takeguchi, Kohno and Sih, 1971), human skin (Ziboh, 1973), guinea-pig lung (Parkes and Eling, 1974), rabbit kidney medulla (Rose and Collins, 1974; Schwartzman, Gafni and Raz, 1976; Tai, Tai and Hollander, 1976), and non-pregnant uteri of cows and guinea-pigs (Wlodawer, Kindahl and Hamberg, 1976). In addition to finding the inhibitor to be heat sensitive, Takeguchi *et al* (1971); Rose and Collins (1974) and Wlodawer *et al*, (1976), all found their respective inhibitors to inhibit in a dose-dependant manner. In these studies the possibility of dilution of labelled substrate by endogenous AA leading to an apparent inhibition was not checked except in the experiments of Wlodawer *et al* (1976) who estimated AA conversion by oxygen uptake on oxygen electrode which eliminated this possibility.

The evidence presented in this study eliminates the possibility that the inhibitory activity was due to simple dilution of labelled substrate. It is then tempting to speculate that the inhibitor is a proteinaceous molecule, as it is heat labile as were others (Takeguchi *et al*, 1971; Tai *et al*, 1976). The inhibitor could have two possible sources of origin, one is the actual cells of the tissue; the other from trapped blood elements. In this context it is worth mentioning that White and Glassman (1976) found that the addition of platelet-poor plasma to washed platelets caused a strong inhibition of PG synthesis from radio-labelled AA. They concluded it was due to the substrate binding onto albumin. More recently Saeed,

MacDonald-Gibson, Cuthbert, Copas, Schneider, Gardiner, Butt and Collier (1977) reported the presence of an endogenous inhibitor of PG synthesis which is a protein, as it is associated with the α -globulin fraction of plasma. If the inhibitor is present in the plasma, which infiltrates virtually every organ in the body it would explain why it can be found in the microsomal supernatants of so many different tissues.

There are conceivably two possible mechanisms by which such an inhibitor could act. The first is that the inhibitor is a large protein and the substrate is bound to it, therefore denying it to the PG synthetase. Serum albumins have been shown to do this (Tai et al, 1976; White and Glassman, 1976). The second mechanism is that the inhibitor blocks the access of substrate to the active site of the cyclo-oxygenase, presumably by reacting with one or more of the active groups. Evidence against the first mechanism has been presented by Harrowing and Williams (1979) who showed that preincubating the microsomal supernatant with the substrate did not increase the amount of inhibition seen and argues against the inhibitor binding the substrate.

General Discussion

Although there is strong evidence that uterine PGs play an active role in parturition (Aiken, 1972; Chester et al, 1972; Hertelendy, 1973; Lewis and Schulman, 1973; Novy et al, 1974; Vane and Williams, 1973) many of these studies have relied upon the use of PG synthetase inhibitors such as indomethacin. This type of experiment does not allow any conclusion as to which of the AA metabolites played a role in regulating uterine motility. The experiments reported here have shown some interesting facts, firstly the myometrium produces large amounts of 6-oxo-PGF_{1 α} and the decidua a mixture of PGs. The oxytocic effects of PGE₂ and PGF_{2 α} have been well documented, however the question arises, if decidual PGs play a role in governing uterine motility how do they pass from the decidua to the myometrium? A process of diffusion would have to be invoked and as this would not proceed preferentially in either direction then much of the decidual PGs would be expected to diffuse intra-luminally and pass into the amniotic fluid. Thus the actual concentration reaching the myometrium would be considerably reduced. It is a much more attractive hypothesis that the myometrium itself produces a substance which governs its contractility. As yet very little is known about the actions of prostacyclin on the reproductive organs. It does appear that prostacyclin is a weaker oxytocic than either PGE₂ or PGF_{2 α} (Omini, Moncada and Vane, 1977; Williams, El Tahir and Marcinkiewicz, 1979). However, prostacyclin in subthreshold doses will potentiate oxytocic-induced uterine contractions (Williams et al, 1979). This could mean that the increase in myometrial prostacyclin formation during the last days of pregnancy (Williams, Dembinska-Kiec, Zmuda and Gryglewski, 1978) progressively sensitizes the myometrium to circulating oxytocin (Williams et al, 1979).

Recently Omini, Falco, Passargiklian, Fano and Benti (1979) have demonstrated that prostacyclin lowers uterine tone and reduces spontaneous contractility in isolated strips of human pregnant myometrium. They postulate that prostacyclin may modulate uterine motility by this action. But a conflicting report has also recently been published showing that prostacyclin has an oxytocic effect on the human pregnant myometrium (Wilhelmson, Lindblom, Hamberger, Samsioe, Hammarstrom, Wiggvist and Samuelsson, 1979) and so at the present stage it is difficult to say whether prostacyclin has mixed actions in the human myometrium. The discrepancy between the different groups could be due to the different areas of the uterus from which the tissue samples were taken. There is also the possibility as seen in the rat that as prostacyclin is a weaker oxytocic than PGs it may be produced in order to dilute the effects of the more potent oxytocics produced in the decidua. But as to why one should see a large increase in myometrial prostacyclin formation at term (Williams et al, 1978) along with a large increase in prostaglandin synthesis remains to be elucidated.

Apart from an active role at the time of parturition in influencing contractility it must be in mind that 6-oxo-PGF_{1α} and hence by inference prostacyclin is the major product of the decidua and the myometrium between days 13-22 of pregnancy when the foetus is rapidly developing. It is possible that prostacyclin so produced may play other roles. One suggested by Williams et al (1978) was that myometrial prostacyclin may help to maintain an adequate uterine blood supply to the developing foetus as prostacyclin has been shown to be a potent vasodilator in the rat (Armstrong, Lattimer, Moncada and Vane, 1978).

It is possible that the low synthetic activity seen with microsomal preparations of MUP and placentae could well be due to the action of an inhibitor. A possible role for an endogenous inhibitor is not hard to find. As prostacyclin is a potent vasodilator (Armstrong et al, 1978) suppression of its production at the site of placental attachment would be logical as blood loss after placental detachment would be reduced. A further function would be to suppress localised contractions beneath the placentae, thus preventing premature detachment. The finding that similar inhibitors are present in tissues other than the placenta indicates that they could play a role in local control of PG synthesis throughout the body.

Conclusions

1. Rat uteri on day 22 were separated into constituent tissues, decidua and myometrium. The AA metabolism by microsomes from these tissues was studied using a radiometric assay. Products were separated and identified using various TLC procedures.
2. The major AA metabolite synthesized by myometrial microsomes was 6-oxo-PGF_{1α}. The decidual microsomes were found to synthesize PGE₂, 6-oxo-PGF_{1α}, PGF_{2α}, PGD₂ and TXB₂. PGE₂ was the major product.
3. Optimal incubation conditions for decidual and myometrial PG synthetase are similar but not identical to other PG synthetase systems. There are also differences between decidual and myometrial PG synthetases, particularly in regard to synthetic capacity and optimal substrate concentration.

4. PG synthesis by decidual and myometrial microsomes is absolutely dependent on the inclusion of an anti-oxidant in the incubation medium.
5. The ratio of PG-like products synthesized by either decidual or myometrial microsomes was unaffected by the presence or absence of thiol containing cofactors (e.g. GSH and L-cysteine). However, overall synthesis was decreased in the absence of thiol containing cofactors in both preparations.
6. Substitution of adrenaline, noradrenaline, 5-hydroxytryptamine, histamine and L-ascorbic acid for hydroquinone as a cofactor significantly increased $\text{PGF}_{2\alpha}$ synthesis in decidual microsomes, but 5-hydroxytryptamine was the only cofactor which was as effective as hydroquinone in stimulating total PG production. Substitution of hydroquinone by adrenaline, noradrenaline or 5-hydroxytryptamine led to an overall stimulation of PG synthesis in myometrial microsomes, but did not affect the ratio of products formed.
7. PG synthesis by decidual and myometrial microsomes was inhibited by a variety of NSAIDs. The most potent of these was found to be flurbiprofen.
8. As pregnancy progresses the PG synthesizing capacity increases in both decidual and myometrial microsomes. Maximum synthetic capacity occurs on day 22 of pregnancy, day of delivery, and declines sharply within 24 hours post-partum.
9. There is little PG synthetic activity in microsomal preparations of MUP and placenta taken from day 22 rat uteri.

10. An inhibitory factor(s) is present in the microsomal supernatant fraction of microsomal preparations of decidua, myometrium and placenta. This factor(s) is heat labile, inhibits PG synthesis in a dose-dependent fashion and acts at the cyclooxygenase level. Its identity and chemical nature have yet to be elucidated.

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Differential prostaglandin production by microsomal fractions of rat pregnant uterus

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Crude homogenates of decidual tissue from rat pregnant uteri synthesize prostaglandin (PG) F as estimated by parallel bioassay (Williams, Sneddon & Harney, 1974). As recent work has shown the increasing complexity of arachidonic acid metabolism we have developed a radiometric assay to allow more detailed analysis of products formed by the uterus.

Pregnant rats were killed (day 22 of pregnancy) and 20% homogenates of deciduum and myometrium prepared in Tris/acetate buffer (pH 8) containing hydroquinone 0.5 mM and reduced glutathione 2 mM. Microsomes were prepared by ultracentrifugation and samples incubated with 1 ml aliquots of buffer containing arachidonic acid (99 nmol) and [14 C]-arachidonic acid (3.5 nmol) for 30 min at 37°C. Pilot experiments indicated these conditions were optimal for decidual tissue with regard to substrate and co-factor concentrations, pH and incubation time. Samples boiled before incubation served as controls. After solvent extraction residues were subjected to thin-layer chromatography (TLC) using the solvent chloroform 90: methanol 9: acetic acid 1: water 0.65 by volume. The radioactivity in consecutive 0.5 cm bands of each zone was then estimated by liquid scintillation counting. Authentic $\text{PGF}_{2\alpha}$, PGE_2 , [14 C]-6-oxo $\text{PGF}_{1\alpha}$ (prepared as described by Cottee, Flower, Moncada, Salmon & Vane, 1977) and arachidonic acid were used as markers.

With decidual microsomes 23% of radioactivity on the TLC plate was converted to products other than monohydroxy acids (range 14–50%, 15 experiments). 50% of this radioactivity had an R_F value similar to PGE_2 , 25% ran as PGD_2 while 20% had a mobility equivalent to $\text{PGF}_{2\alpha}$. Myometrial substrate conversion was lower, 6% (range 2–15%, 15 experiments) and all the radioactivity was located in the PGE_2 zone. However, in this solvent system PGE_2 and 6-oxo $\text{PGF}_{1\alpha}$ have similar R_F values. Therefore the zones of radioactivity corresponding to PGE_2 were removed, eluted and rechromatographed using solvent systems devised to separate these two substances (Cottee *et al.*, 1977). Using these TLC systems we found that approximately 12% of radioactivity in the decidual PGE_2 zone behaved as 6-oxo $\text{PGF}_{1\alpha}$ and 70% as PGE_2 . However 84% of myometrial radioactivity appeared to be 6-oxo $\text{PGF}_{1\alpha}$ and only 6% PGE_2 .

Thus decidual microsomes produce PGE_2 as the major product whereas myometrial preparations synthesize predominantly 6-oxo $\text{PGF}_{1\alpha}$. Further investigation is needed to determine whether this myometrial PG production plays any part in regulating uterine function during pregnancy.

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PROSTAGLANDIN AND THROMBOXANE PRODUCTION
BY RAT DECIDUAL MICROSOMES

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ABSTRACT

The preparation of a microsomal fraction from the decidual tissue of pregnant rat uteri is described. Incubation of such microsomes with a mixture of radiolabelled and cold arachidonic acid (51 μ M) plus co-factors resulted in a 30% substrate conversion. Products were resolved into four peaks (A,B,C and D) by thin-layer chromatography. Combined gas-liquid chromatography-mass spectrometry and further thin-layer chromatography identified the products as $\text{PGF}_2\alpha$ (A); thromboxane B_2 (B); a mixture of 6-OXO $\text{PGF}_1\alpha$ and PGE_2 (C); PGD_2 and PGE_2 (D). PGE_2 was the major product.

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INTRODUCTION

Many experimental findings suggest that prostaglandins (PGs) play an important role in parturition. Williams and Vane [1] have reviewed the evidence favouring PG involvement. Many experiments have depended upon the use of non-steroidal anti-inflammatory drugs as PG synthetase inhibitors. For instance indomethacin blocks both the spontaneous contractions and PG release from the rat pregnant uterus *in vitro* [2,3] and delays parturition and prolongs labour in rats [3]. As a result of such findings indomethacin has been used clinically as a rational treatment for inhibiting uterine contractions in premature labour [4]. Despite the clinical importance of blocking uterine PG synthesis little is known of the PG synthesizing system in the pregnant uterus. As a first step in such a study we have used a microsomal preparation from the decidual tissue of the pregnant rat uterus as our source of PG synthetase. This study describes the products which are formed when this enzyme system is incubated with arachidonic acid.

METHODS

Female rats (CFY strain) were killed on day 22 of pregnancy (expected day of delivery). Decidual tissue was separated from the myometrium by scraping. A 20% ($\frac{w}{v}$) decidual homogenate was prepared in ice-cold 0.1 M Tris/acetate buffer (pH8) containing sucrose (0.25M), EDTA (1mM), hydroquinone (0.5 mM), L-cysteine (1mM) and reduced glutathione (2mM) using an Ultra-Turrax homogeniser. The homogenate was

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centrifuged at 3000 x g for 30 minutes and the supernatant recentrifuged at 100,000 x g for 60 minutes. The microsomal pellet was resuspended in buffer by ultrasonication (20KHz, 150W for 15 seconds) to give a protein concentration of 1-2 mg/ml. 1 ml aliquots of the suspension were incubated for 30 minutes at 37°C with 1ml of buffer containing arachidonic acid (99 nmol) and [$1-^{14}C$] arachidonic acid (200 nCi) giving a total substrate concentration of 51 μ M. The reaction was terminated with 0.25ml of 2M citric acid. Samples boiled for 60 seconds before incubation acted as controls. Acidified samples were extracted twice with 2 volumes of ether. In the majority of cases the combined extracts were dried using anhydrous sodium sulphate before evaporation under nitrogen and immediate addition of 40 μ l of chloroform. Each sample was then applied to thin-layer chromatographic (TLC) plates (silica gel G, 0.25 mm thickness) followed by authentic PGE₂, PGE₁ and arachidonic acid, in some experiments PGD₂, PGA and PCB were also applied. The plates were developed in chloroform, methanol, acetic acid and water (90:9:1:0.65) to 15 cm. After drying authentic markers were visualised with iodine vapour. Areas of radioactivity were detected using a Berthold TLC scanner. Each zone was then divided into 0.5cm bands which were scraped off and the radioactivity eluted with 1ml of methanol. 9ml of scintillant (0.5% PPO and 0.03% dimethyl POFOP in toluene) were added and radioactivity determined by liquid scintillation counting. Absolute activity (d.p.m.) was calculated by the counter using a pre-programmed quench correction curve based on a channels ratio.

In several experiments the C and D peaks (see results) were scraped off, eluted with methanol and resolved in other TLC systems. Peak C was resolved in (a) the organic (upper) phase of ethyl acetate iso-octane, acetic acid, water (11:5:2:10) on plates described above or (b) chloroform, methanol, acetic acid (20:10:10) on TLC plates impregnated with 5% silver nitrate. These systems were devised to separate PGE₂ and 6-oxo PGE₁ [5]. Peak D was rechromatographed using ether, methanol, acetic acid (90:1:2) which separates PGE₂ and PGE₁ [6]. In these experiments PGE₂, PGE₁, PGD₂ and [$1-^{14}C$] 6-oxo PGE₁ were used as markers.

The preparation of 6-oxo PGE₁ has recently been described [5]. 100 nCi of [$1-^{14}C$] arachidonic acid were incubated with 10 ng of ram seminal vesicle microsomes in 1 ml of Tris buffer (pH 7.4) for 15 minutes at room temperature. The [$1-^{14}C$] 6-oxo PGE₁ was separated by TLC using the systems described above.

Gas-Liquid Chromatography and Mass Spectrometry (GLC-MS)

A preparative synthesis using 30 ng. of decidual microsomes was carried out (incubated in 6 x 5 ng amounts as described). After TLC the zones containing radioactivity were detected by scanning, scraped off and eluted with methanol. From each sample the methyl ester, methyl oxime, trimethyl silyl ether was prepared. Products were identified by GLC-MS and quantitated against the derivative of authentic PGE₂. Full details of the GLC-MS procedure have been published [7].

RESULTS

The distribution of radioactivity on a TLC plate after incubation of decidual microsomes with labelled arachidonic acid is shown

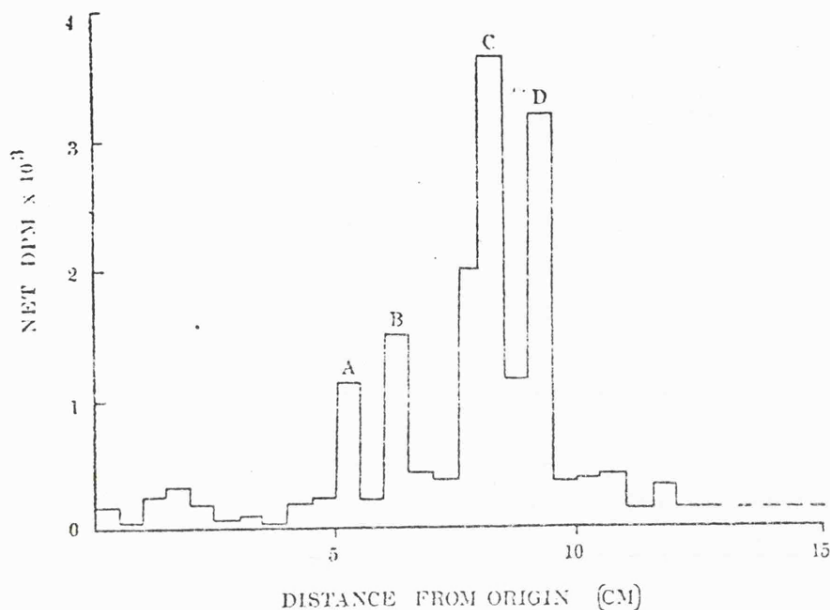


Figure 1. Distribution of radioactivity on a TLC plate after incubation of decidual microsomes with radiolabelled arachidonic acid. Net d.p.m. indicates test minus boiled control value. The broken line at the solvent front indicates more radioactivity present in the boiled control zones.

In Figure 1. Four distinct peaks were seen which were not present in boiled control samples. Peak A had an R_F value similar to authentic PGF_1 (0.35) with peak B running slightly higher (R_F 0.42). Peak C had a mobility equivalent to authentic PGE_2 (R_F 0.56). The least polar peak D had an R_F of 0.61. Substrate conversion was 23% in this experiment and a mean of 10% (range 15-55) in 17 experiments. Product formation (substrate converted) was taken as the radioactivity between peaks A and D expressed as % of the total activity recovered from the plate (after subtraction of boiled control values). This figure thus excludes monohydroxy fatty acids produced which run close to arachidonic acid in this system. PGA and PGB were not included as these substances have R_F values of 0.80-0.85 in this system and negligible amounts of these products were noted.

In Figure 1 the contribution of each peak to the total product was as follows: peak A 10%; peak B 13%; peak C 50%; peak D 27%. Subsequent chromatography of peak C in the first solvent system indicated the presence of two products with R_F values corresponding to 6-oxo PGF_1 (R_F 0.12) and PGE_2 (R_F 0.24). This was confirmed using the second solvent

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system with nitrated plates PGE_2 (R_f 0.44), 6-oxo $\text{PGF}_1\alpha$ (R_f 0.70). In these experiments the zone corresponding to PGE_2 contained the majority of radioactivity, 74% (range 64-86 $n = 6$); 9% (range 4-16 $n = 6$) of the label had a mobility similar to 6-oxo $\text{PGF}_1\alpha$.

Peak D was also resolved into two peaks with mobilities equivalent to PGE_2 (R_f 0.16) and PGD_2 (R_f 0.42.) The two products were present in similar quantities, PGE_2 zone 39% (range 27-49 $n = 6$) and PGD_2 zone 41% (range 21-55 $n = 6$).

GLC-MS was used to analyse the contents of the four peaks. Peak A contained the major ions associated with $\text{PGF}_2\alpha$ and constituted 11% of the estimated product. Peak B was identified as thromboxane B_2 (3%). Peak C contained the major ions of 6-oxo $\text{PGF}_1\alpha$ (12%) and PGE_2 (69.5%). Peak D contained PGE_2 (4.5%). No PGD_2 was detected in the extract.

DISCUSSION

These results confirm earlier findings which indicated that decidual tissue from the pregnant rat uterus at term has a high PG synthesizing capacity [8]. Chromatographic procedures indicated the presence of five products with mobilities similar to PGE_2 , PGD_2 , $\text{PGF}_2\alpha$, 6-oxo $\text{PGF}_1\alpha$ and thromboxane B_2 . Subsequent GLC-MS confirmed the presence of all the products except PGD_2 . The relative proportions of the substances estimated by the two methods agreed well taking into account that no correction for loss was made through the derivatisation procedure for GLC-MS. Also quantitation of all products against $\text{PGF}_2\alpha$ is not ideal but was used due to lack of authentic 6-oxo $\text{PGF}_1\alpha$ and thromboxane B_2 . The reason for the lack of major ions associated with PGD_2 is unclear (the derivative of PGD_2 was subjected to GLC-MS and a satisfactory spectra obtained). The GLC-MS result however is from only one experiment, using TLC procedures a peak with an R_f similar to PGD_2 is seen consistently. The presence of PGE_2 in the D peak is probably due to overlapping caused by inadequate separation of PGE_2 and PGD_2 by this solvent system.

Crude uterine homogenates synthesize predominantly $\text{PGF}_2\alpha$ as estimated by bioassay [2] whereas the microsomal preparations produced PGE_2 in the largest amounts. This difference is not accounted for by co-factor additions to the microsomes as incubations without glutathione did not affect substrate conversion whereas no synthesis took place in the absence of hydroquinone (unpublished observations). The factors governing the formation of individual PGs by the uterus is obviously complex as under the incubation conditions used myometrial microsomes produce mainly 6-oxo $\text{PGF}_1\alpha$ [9]; this is also the major product of homogenates of pseudopregnant rat uterus [10].

6-oxo $\text{PGF}_1\alpha$ and thromboxane B_2 synthesized by decidual microsomes are the stable products formed from the unstable prostacyclin (PGI_2) and thromboxane A_2 respectively [11,12]. Whether these biologically active intermediates or their end products exert any actions in the pregnant uterus remains to be elucidated. Further experiments are in progress to investigate the factors controlling arachidonic acid metabolism and to determine the relative contributions of the products to uterine function during pregnancy.

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